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individual field effects appear to be additive for respiration but not for nuclear division rate. For fields applied simultaneously at levels below 0.14 V/m and 0.4 G the response was independent of field intensity. No threshold was observed for simultaneously applied electric and magnetic fields; however, indirect evidence is presented that suggests either the electric or magnetic field is below threshold at levels of 0.14 V/m and 0.4 G respectively. Frequency modulation of the fields seems to have no major effect on the response induced in P. polycephalum.

Results of additional experiments are presented that are designed to rule out certain non-EMF factors as being responsible for the biological changes. Factors tested include ambient electromagnetic fields, incubator differences electrolytic effects and investigator bias.

TABLE OF CONTENTS

Goodman, E. M., Greenebaum, B., and Marron, Michael, T., Bioeffects of Extremely Low Frequency Electromagnetic Fields: variation with intensity, waveform and individual or combined electric and magnetic fields. Radiat. Res.

Exposure of the myxomycete Physarum polycephalum to either continuous wave (75 Hz) or frequency modulated wave (76 Hz) electromagnetic fields (EMF) (0.1 G to 2.0 G and 0.035 V/m to 0.7 V/m) lengthens the mitotic cycle and depresses the respiration rate. Once induced, these effects persist indefinitely without increasing or decreasing in magnitude beyond that due to normal variability of the organism. Similar effects are observed when either individual electric fields (0.7 V/m) or magnetic fields (2.0 G) are applied, however the magnitude of the response is less than that observed with simultaneous fields. The individual field effects appear to be additive for respiration but not for nuclear division rate. For fields applied simultaneously at levels below 0.14 V/m and 0.4 G the response was independent of field intensity. No threshold was observed for simultaneously applied electric and magnetic fields; however, indirect evidence is presented that suggests either the electric or magnetic field is below threshold at levels of 0.14 V/m and 0.4 G, respectively. Frequency modulation of the fields seems to have no major effect on the response induced in P. polycephalum.

INTRODUCTION

The research described in this report represents the second phase of a program designed to ascertain the effects of extremely low frequency electromagnetic fields (ELF) on the slime mold Physarum polycephalum. The first phase (see Technical Report, Phase 1, 9/15/71-6/30/74) involved exposure of Physarum at various phases of its life cycle to continuous wave fields of 75 and 60 Hz, 2.0 G, and 0.7 V/m. The data from the studies indicated a lengthening in the mitotic cell cycle and a slowing of shuttle streaming; the competency of the mold to complete its life cycle was not adversely effected by exposure to these fields. If the affected cultures were removed from the ELF environment and returned to a control environment, length of the cell cycle returned to control levels within about 3 to 4 weeks.

This report examines the roles of individual electric and magnetic continuous wave (cw) fields in addition to the effects of frequency-modulated fields on the nuclear division cycles. ELF effects on various physiological and biological parameters are also discussed.

MATERIALS AND METHODS

Field Generation and Monitoring: Magnetic and electric fields were generated using facilities and equipment described in detail elsewhere (11,12). Briefly, magnetic field coils surround both a reciprocating platform (to aerate microplasmodia in flasks), and a stationary platform (for macroplasmodia in petri dishes); both shaker and coils are enclosed in a Warren-Sherer RI-48 incubator. Electric fields are generated by stainless steel electrodes placed in contact with the nutrient medium. Control and experimental incubators are similar in all respects except that coils and electrodes in the control

incubator are not energized. In those experiments in which electric and magnetic fields are applied simultaneously both the coils are energized. In experiments involving a single field (either electric or magnetic) only the electrodes or the coils are energized; the power connection to the unused circuitry is broken and replaced with a dummy load with the same impedance as the power amplifier. Oscillating 75 Hz electric and magnetic fields were applied using a sine waveform with the fields at right angles to one another and in phase. In several experiments the waveform was modulated by shifting the frequency abruptly at the peaks so as to maintain a continuous waveform; this type of frequency modulation is known as minimum-shift-keying modulation and is employed in certain communication systems. In these experiments the oscillator driving the field generation equipment was set at a nominal center frequency of 76 Hz. It made random frequency shifts between 72 Hz and 80 Hz on the average of 8 times a second. The oscillator was furnished by the Illinois Institute of Technology Research Institute under a contract with the Office of Naval Research.

Maintenance of Cultures and Mitosis Experiments: Physarum polycephalum M_3C_{VII} (IIe) originally obtained from Dr. Joyce Mohberg, McArdle Laboratory for Cancer Research, Madison, Wisconsin, has been continuously maintained and subcultured as microplasmodia in specially designed rectangular shake flasks (12) using the medium described by Daniel and Baldwin (13). At the beginning of our study on the effects of EMF fields on Physarum (September, 1971), microplasmodia were subdivided into four lines; these were designated as controls to which no electric or magnetic fields were ever applied. These microplasmodia have continued to serve both as controls and as the source of inoculum for each experiment concerned with the effects of a new electromagnetic environment. Control and experimental cultures are

maintained in separate incubators with interconnected temperature controls, allowing incubators to be maintained at 25.5 ± 0.3° C (14).

Maintenance of Cultures: In our previous reports (10-12) interpretation of some experiments was complicated by extensive scatter in the data. To improve the precision of our experimental protocols, we found it necessary to rigidly adhere to the following details for handling and culturing Physarum. Microplasmodia from the control and EMF environments are routinely subcultured to new nutrient medium on a rigid 48 hour/48 hour/72 hour schedule. With the exception of the short period of time required for transfer of cultures to fresh growth medium exposed cultures are maintained in the EMF environment at all times. Cultures are prepared for measurement of nuclear cycle length or oxygen uptake rate by withdrawing a 2.0 ml aliquot of microplasmodia from the appropriate stock culture and transferring it into 20 ml of nutrient medium. These new cultures are allowed to grow for only 24 hours whereupon they are processed as described below. This procedure insures that microplasmodia are in the logarithmic phase of their growth cycle at the time measurements are performed.

Nuclear Division Cycle: Logarithmic-phase microplasmodia from both the control and exposed flasks are collected in tared conical tubes and centrifuged for 30 seconds at 250 x g, the supernatant is decanted and the microplasmodial pellet is washed and resuspended in 10 volumes of distilled water. The suspension is recentrifuged (30 s, 250 x g), the supernatant is again decanted, and the pellet is weighed and resuspended in twice the pellet weight of distilled water. A 0.2 ml aliquot of the microplasmodial suspension is placed on filter paper supported by an absorbent cotton pad in a Petri dish. These microplasmodia coalesce to form a single multinucleate syncytium referred to as a stationary

macroplasmodium; prolonged EMF exposure does not affect the fusion of microplasmodia (15). The time required for each stationary macroplasmodium to attain the second metaphase configuration (includes the interdivision time from addition of medium to M_I and the interdivision time between M_I to M_{II}) is referred to as the mitotic cycle. All nuclei in a single stationary macroplasmodium undergo mitosis in virtual synchrony (16). An experiment usually consists of 10 control plates, derived from microplasmodial cultures grown in the control environment, and 10 experimental plates, derived from microplasmodial cultures continuously subjected to the particular field conditions being studied. The stages in the mitotic cycle are determined by observing ethanol-fixed smears with a phase-contrast microscope. After determining the onset of mitosis (metaphase) in each stationary macroplasmodium the cultures are discarded.

Oxygen Consumption: Control and EMF-exposed microplasmodia are collected in the logarithmic phase of their growth cycle by centrifugation at 250 x g(4° C). The supernatant is decanted and the pellet resuspended in an equal volume of fresh growth medium. Duplicate 0.5 ml plasmodial samples from each set are placed in sterile Warburg reaction vessels that have been calibrated according to Umbreit et al. (17). Growth medium (2.5 ml) is added and the flasks are brought to 25.0 $\pm$ 0.1° C in the Warburg water bath. The CO_2 evolved is trapped in the center well using filter papers saturated with hyamine hydroxide (18). At the conclusion of the experiment, protein content is estimated by removing plasmodia from each Warburg vessel, extracting the pigment in several washes of trichloroacetic acid (TCA)-acetone-water (50 gr. TCA in 500 ml acetone and water to one liter) and dissolving the residual pellet in 0.4 NaOH. Protein content in the pellet was estimated using the colorimetric procedures described by Lowry et al. (19) with bovine serum albumin as a standard.

Statistical Methods: Two statistical tests are employed for analyzing the data: the t-test and the Wilcoxon Signed Ranks test, also referred to by some authors as the Mann-Whitney test (20). We routinely apply both tests in our analysis; a difference between the results of the two tests indicates that the distributions are not normal. In this situation the Wilcoxon test is the preferred test.

There is a fairly large day-to-day variation observed in the mitotic cycle lengths and in other features of the Physarum life cycle. This variation is believed to be caused by changes in handling procedures and in uncontrolled external factors such as humidity, barometric pressure, and impurities in the nutrient medium, all of which affect both control and exposed cultures in an identical fashion. Because of these variations, data collected for control and exposed cultures on a given day are treated as paired groups. The average value of the control data is subtracted from each of the measurements taken on the exposed cultures before they are plotted or analyzed. We present these relative data in the form of histograms. If there is no difference between control and exposed cultures, the histogram will be centered at 0.0. The width of the histogram provides a measure of both the natural variability of Physarum and variability in the parameter measured, induced by the presence of EMF fields. This "daily" variability is distinct in nature from "day-to-day" variability caused by changes in external factors.

When cultures are first placed into an exposure chamber they behave in a manner indistinguishable from the controls for a period of time ranging from one to twelve weeks. The precise length of this induction period is difficult to assess because there are no widely accepted criteria for establishing the "onset" of an effect in the mitotic cycle. We adopt the arbitrary criterion of waiting until three successive measurements (on three

different days) reveal a difference in the length of the mitotic cycle significant at the $p = 0.01$ level, before we claim an effect. We observe that after an initial induction period defined in this way, the exposed exhibit a mitotic cycle that is more-or-less constant in length and longer in time than control cultures. All data used in the calculations reported here were collected during the post-induction period. No data were excluded; specifically, EMF exposed cultures showing shorter intermitotic times than controls were included.

RESULTS

We observe a lengthened mitotic cycle in Physarum polycephalum exposed to EMF fields. Typical data are shown in Figure 1 for control and exposed cultures for the period of 400 days during which the experiments in this paper were conducted. The particular line of cultures for which these data were taken had already been exposed to EMF fields for approximately four years which accounts for the numbering of the days on the abcissa. Figure 2 is a histogram of the data from Figure 1 showing differences for exposed cultures relative to the daily control average. The histogram reveals an average mitotic cycle for exposed cultures that is 0.64 hr longer than the control mitotic cycle.

Figures 3, 4, and 5 show the effect on the mitotic division cycle induced by exposure to 75 Hz magnetic fields of 2.0 G (Fig. 3), to 75 Hz electric fields of 0.7 V/m (Fig. 4), and to simultaneous electric and magnetic fields 5 times weaker than those employed for the data presented in Fig. 2 (Fig. 5). The data in each of the histograms are pooled from duplicate sets of experiments, i.e., data from two groups of exposed cultures is displayed, each was introduced into the fields at different times. Tests for reproducibility of our data using data from these duplicate sets are presented in the Discussion.

Data for cultures exposed to electric and magnetic fields in which the waveform is modulated are shown in Figures 6, 7, and 8. The modulated field intensities in Figures 6 and 7 were selected to agree with the cw field intensities used for Figures 2 and 5. Figure 8 presents data taken at field intensities attenuated by an additional factor of 4. Thus the fields employed in the set of experiments reported in Figure 8 are a factor of 20 times weaker than those employed for Figures 2 or 6.

The effects of various EMF fields on oxygen consumption are shown in Fig. 9. Differences in rates for exposed cultures from the daily control culture average are presented as percentages. If there were no overall or systematic differences in the respiration rate the histograms would be centered around zero. A decreased respiration rate is observed in all cultures exposed to EMF fields; the magnitude of the decrease in rate varies with exposure conditions. Mean values of the decreases computed from the raw data are given on the righthand side of the Figure. These mean values are all significantly different from zero; the precise level of significance, determined using the Wilcoxon Signed Ranks statistic, is also given in the Figure. The least significant decrease is found in the MOD (E+B) data where a significance level of $p = 0.03$ is determined.[2] The average absolute value for the control respiration rate was 0.6 µl O_2/min/mg-protein; day-to-day variations in control rates ranged between 0.4 and 0.8 µl O_2/min/mg-protein. As we noted above in connection with the mitotic cycle length measurements, day-to-day variations were observed to occur in both exposed and control cultures; whenever high (or low) rates were observed in control cultures correspondingly high (or low) rates were also observed in exposed cultures. It should be noted that day-to-day variations do not affect the precision of our experiments.

ANALYSIS AND DISCUSSION

We have previosly shown that long-term exposure of Physarum polycephalum to continous wave extremely low frequency fields (EMF) of 45, 60, and 75 Hz at 2.0 G and 0.7 V/m lengthens both the mitotic cycle and slows the shuttle streaming of the cytoplasm (10, 11). Here we examine individual electric and magnetic fields in addition to the effects of changing the waveform from sinusoidal to a frequency modulated waveform. An improvement in handling procedures (see methods) was undertaken near day 1500 (Fig. 1), most of the data reported here were collected after this time. A major difference in the data reported here and that reported earlier (10, 11) is that laboratory procedures for handling and transferring cultures have been further modified so that daily and day-to-day variability in the data has been substantially reduced. Daily mitotic cycle lengths were usually determined to within a standard error of less than 0.1 hr. A tabular array of some typical raw data has been published elsewhere (21).

One feature of EMF exposures we were concerned about was the effect of long-term, chronic exposure. Figure 2 presents data for field conditions that are the same as those previously reported in Ref. 11; however, these are new data acquired by continuing our earlier experiments. Cultures maintained in E+B fields[2] have displayed a lengthened mitotic cycle at a nearly constant level, relative to controls, for almost five years. Application of individual magnetic fields (2.0 G, Fig. 3) or electric fields (0.7 V/m, Fig. 4) induces a lengthened mitotic cycle, the magnitude of which is not as large as that observed when both fields are applied simultaneously. If magnetic and electric fields are simultaneously applied at intensities five times weaker (0.4 G and 0.14 V/m), the mitotic cycle is again lengthened about the same amount as that observed when individual, more intense E or B fields, are applied. These data may be

interpreted in one of two ways:

(1) We have observed a typical dose-response relationship since decreasing the intensity by a factor of five (E+B $\tilde{E}+\tilde{B}$) has produced a decreased response.

(2) Alternatively, the decreased response observed in going from E+B to $\tilde{E}+\tilde{B}$ is the result of passing the lower threshold for either the electric or the magnetic field and the efficacious field produces the same response at the reduced intensity as it does at the higher intensity.

Our protocol did not allow for an examination of lower B fields and thus we are unable to provide an unequivocal distinction between these two interpretations. However our data suggest that the second interpretation is more likely. The fact that the magnitude of the effect observed is the same for all three exposure conditions, E-only, B-only, and $\tilde{E}+\tilde{B}$, is easily understood using the second interpretation. Furthermore, experiments discussed below using modulated waveforms, show no alteration in response when field intensities are decreased by another factor of four from $\tilde{E}+\tilde{B}$. This is consistent with the thesis that the remaining efficacious field produces a response that is independent of intensity near these levels.

To make objective comparisons between distributions of data one must adopt some criterion for determining differences based on a statistical test. There are a number of ways of comparing two distributions of numbers, the most common of which are to examine differences in the means or to examine differences in the variances (widths) of the distributions. It is clear that differences exist in the variances of these distributions, however, we have not adopted a test based on this feature of the distribution because we are unsure what biological interpretation to attach to a broad versus a narrow distribution of mitotic cycle times. Presumably a broader

distribution reflects action of an agent that increases the variability in cycle length over and above the natural variability. The test we have adopted is the Wilcoxon Signed Ranks method, and, because it is widely known, we have also applied the t-test for comparison of means in several instances. Both tests are used either to compare two distributions of data to see whether their means differ (it is the distribution locations that are compared) or to determine if the mean of a distribution differs from zero. The Wilcoxon test is a nonparametric test, which means that the distribution being analyzed need not be a normal distribution for the test to be valid. Generally, the two tests produce similar findings; in those instances where a discrepancy exists, a departure from normality in the data is indicated and the Wilcoxon test is preferred.

Before proceeding with a comparison of distributions of mitotic cycle lengths it is important to examine the data for reproducibility and consistency. The data presented in Figure 3 are pooled from two sets of experiments both of which employed 75 Hz magnetic fields at 2.0 G. These experiments were not conducted simultaneously but were staggered in time. After a lengthened cycle was established in the first set of cultures exposed to the fields, a second set of cultures derived from the control was introduced into the field generation apparatus. Figures 4 and 5 likewise present data pooled from two sets of cultures. Reproducibility in these data may be tested by constructing distributions for each individual set of cultures and comparing them with one another. The results of these comparisons are presented in Figure 10a. The notation we use in this and later figures is set forth in Table 1. The two sets of data for each field condition are distinguished by a subscript "1" or "2" on the field condition symbol. Comparison of the two B-distributions gives a Wilcoxon statistic of 1.1 which is far below the $p = 0.01$ level of significance required for

establishing a difference between distributions. There is no significant difference between the two E-distributions or the two $(\vec{E}+\vec{B})$-distributions. The number (N) of data in each distribution is given in the Figure.

The internal consistency of our data may be examined by comparing the results of equivalent tests such as B_1 vs E_1 and B_2 vs E_1. The first four lines of Figure 10b show that all possible comparisons of B-sub-distributions with E-subdistributions produce the same statistical finding, viz., that the B-distributions are indistinguishable from the E-distributions. The last eight lines of Fig. 10b present all possible remaining tests among the six distributions introduced in Figure 10a. These tests establish the consistency of our data; one reason we selected a significance level of 0.01 was to insure this internal consistency. In the remainder of the Discussion we deal only with pooled distributions.

A statistical comparison of the E+B, B-only, and E-only distributions is shown in Figure 11a. Note that the abcissa scale in Figure 11 has been compressed by a factor of 2 over that in Figure 10. The statistical tests clearly support the conclusion formed by visual inspection that the B-only and E-only distributions are similar and both are different from the E+B-distribution. This means that <u>both</u> the magnetic and the electric fields play a role in causing physiologic changes in the organism. We see no <u>a priori</u> reason that the B-field and E-field should have the same effect. The statistical identity of the B- and E-distributions may arise because of fortuitous selection of two field intensities at appropriate levels to cause this to happen. Note also that although both B- and E-fields each contribute to lengthening the mitotic cycle, their input is apparently not additive when the fields are applied simultaneously.

Figure 11b shows the statistics for comparisons of E+B, B-only, and E-only distributions with the $\vec{E}+\vec{B}$ distribution confirming the visual analysis.

As noted above this finding suggest that by decreasing the E and E field intensities by a factor of 5 we have passed the lower threshold for one of the fields. In an earlier paper we reported that E+B fields did not lengthen the mitotic cycle in Physarum (11). The data presented in Figure 5 clearly contradict the earlier report that was based on different data. Because of large variability in our earlier data, we were unable to reject the hypothesis of no effect. The experimental data presented in Figure 5 were collected subsequent to our first report and after more stringent laboratory procedures for handling cultures had been implemented. The data are reproducible (see Figure 10a) and unequivocal in establishing an effect. Our earlier conclusion that a lower threshold had been passed must be discarded.

The influence that waveform may have in inducing effects in Physarum was also examined. Modulated fields, electric and magnetic, were applied at three different intensities, the least intense being 20 times weaker than the most intense (Figures 6, 7, and 8). Visual inspection of these three distributions shows that they are all quite similar. The major difference is that some observations lie at quite large cycle times in Figure 6, compared to Figures 7 and 8. If it were not for the upper tail in Figure 6, the three would appear almost identical. The statistics for comparisons are given in Figure 11c where it may be seen that the conclusions based on visual inspection are confirmed. The difference between MOD(E+B) and MOD($\check{E}+\tilde{B}$) is not large but it is significant. Agreement between MOD($\tilde{E}+\tilde{B}$) and MOD($\check{E}+\check{B}$) is somewhat surprising, suggesting that Physarum's response to fields at these levels is independent of intensity. One must be careful in drawing negative conclusions: they are only as good as the power of the statistical test applied. Although it is difficult to determine the power of the Wilcoxon test, the power of the t-test is well established and may

be used to estimate the power of the Wilcoxon test. Using t-test power tables (22) one may say with 99% confidence that the true difference in means between the distributions in Figures 7 and 8 is at most 0.01 hr.

The agreement between the MOD(E+B) and $\overset{\approx\approx}{\text{MOD}(\text{E+B})}$ data is somewhat harder to understand. One would intuitively expect the reverse since $\text{MOD(E+B)} \not\approx \text{MOD}(\tilde{\text{E}}+\tilde{\text{B}})$ and $\text{MOD}(\tilde{\text{E}}+\tilde{\text{B}}) = \overset{\approx\approx}{\text{MOD}(\text{E+B})}$. When a set of results is not internally consistent we say that transitivity within the set does not hold. Transitivity means that if A=C and B=C then A=B. There is of course no reason for transitivity of these relations to hold because the equality signs do not represent strict numerical equality but rather statistical indistinguishability. We note that another violation of transitivity arises in connection with the MOD(E+B) distribution, which is seen in Figure 11d. It is tempting to suppose the lack of transitivity may be traced to the long-cycle tail in the MOD(E+B) distribution, though the degree of influence and the physical significance of the tail is not clear.

A comparison of individual modulated field distributions with the unmodulated field distributions is presented in Figures 11d, 11e, and 11f. Except for the lack of agreement between the MOD(E+B) and E-only distributions and the agreement between MOD(E+B) and $\overset{\approx\approx}{\text{MOD}(\tilde{\text{E}}+\tilde{\text{B}})}$ there are no surprises. In fact the findings provide independent confirmation of the conclusions reached as a result of the analyses in Figure 11c. The entire results of Figure 11 are summarized in schematic form in Figure 12.

The effect of EMF on oxygen uptake (Figure 9) is similar to its effect on the nuclear division cycle. The decrease in oxygen uptake for E+B is greater than that for any of the other field conditions. In contrast to the mitotic cycle length data, the B-only and E-only effects appear to be additive, i.e., one-half of the E+B decrease may be ascribed to the B-field and half to the E-field. Again, perhaps fortuitously, the magnitude of the

B-field and E-field effects are nearly identical. U like the mitotic cycle length data, the respiration rate decrease under $\widetilde{E}+\widetilde{B}$ does not agree with B-only and E-only decreases. However, the data for the $\widetilde{E}+\widetilde{B}$ case are few and this conclusion should be accepted with caution. The MOD(E+B) data again stand out from the rest and the distribution is quite broad. No dose-response relationship is observed in comparing MOD($\widetilde{E}+\widetilde{B}$) with MOD($\widetilde{\widetilde{E}+B}$).

The oxygen data in Figure 9 further strengthen the conclusion that waveform has little direct influence on the organismal response to ELF fields. The extent of agreement between the EMF induced differences in mitotic length data and the oxygen uptake data can be seen by examining the means and standard deviations for the two sets of distributions, listed together in Table 2. The longest mitotic cycle length increases parallel the greatest decreases in respiration rates. Broad distributions of cycle lengths are usually associated with broad distributions of respiration rates. Measurements of respiration rates and cycle lengths usually are not performed on the same day. One can readily imagine mechanisms for explaining a close correlation between respiration rates and mitotic cycle lengths. The correlation of distribution widths between the two sets of data presumably reflects the close connection between mitosis rate and respiration rate. Exactly why some distributions are broader than others is not clear.

Although the oxygen uptake experiments seem to be telling us the same thing as the mitotic cycle length experiments when B- and E-fields are applied simultaneously, they tell us something different when the fields are applied individually. The fields seem to be additive in their effects on oxygen uptake and not additive in their effects on mitotic cycle length. This may suggest that there are several mechanisms of interaction between ELF fields and biological systems or, what is more likely, it may simply reflect a complex relationship between respiration rate and mitosis rate.

SUMMARY

1. Exposure of Physarum polycephalum to extremely low frequency EMF electric (0.035 to 0.7V/m) and magnetic fields (0.1 to 2.0 G) produces a decrease in respiration and an increase in the nuclear division rates in the organism. These findings are consistent with results of earlier studies performed at different frequencies and field intensities.

2. Several sets of Physarum cultures have been exposed continuously for five years to 75 Hz fields of 0.7 V/m and 2.0 G. After an initial induction period the exposed cultures exhibited a mitotic cycle that was consistently longer than the control cycle by 0.6 hr. The induced increase in mitotic cycle length does not become progressively larger nor does the organism respond by compensating for exposure and slowly adjust its cycle length to agree again with the control cycle length. The observed decrease in the respiration rate exhibits similar behavior.

3. Application of either a 75 Hz, 0.7 V/m electric field or a 75 Hz, 2.0 G magnetic field produces a decrease in growth and respiration rate; however, the effects are not as large as they are when both fields are applied simultaneously. Either field at these levels produces effects that are statistically indistinguishable from one another. We conclude that both electric and magnetic fields play a role in causing physiologic changes in the organism. It appears that these roles are additive when one examines the respiration data. The roles of the individual fields in slowing nuclear division are not additive: each field causes the rate to slow by about 0.4 hr but simultaneous application of the fields produces a cycle that is only 0.6 hr longer.

4. Exposure of Physarum to simultaneous 75 Hz electric and magnetic fields that are five times weaker (0.14 V/m and 0.4 G) produces effects

that are statistically indistinguishable from those observed when either a stronger electric field (0.7 V/m) or magnetic field (2.0 G) is applied by itself. These and other data suggest that the lower threshold for efficacy of one type of field may have been passed.

5. Frequency modulation of the applied electric and magnetic field produces bioeffects similar to those observed when unmodulated sinewave fields are applied. Although the data obtained are not completely self-consistent, we conclude that frequency modulation of ELF electromagnetic fields at these levels does not substantially alter the way ELF fields interact with biological systems.

6. No lower (or upper) threshold is observed for effects of ELF fields on Physarum. Electric fields were applied ranging from 0.035 V/m to 0.7 V/m; magnetic fields ranged from 0.1 G to 2.0 G. This finding contradicts one from an earlier report that ELF fields of 0.15 V/m and 0.4 G produce no effect in Physarum (11).

7. The dose-response relationship between field intensity and magnitude of the decrease in growth or respiration rate in Physarum is either very weak or nonexistent at the field intensities we have employed. A decrease of field intensities by a factor of four from 0.14 V/m and 0.4 G to 0.035 V/m and 0.1 G produces no significant difference in response. We attribute the large and significant difference in response observed when fields are reduced five times from 0.7 V/m and 2.0 G to 0.14 V/m and 0.4 G as being due to passing the lower threshold for one of the fields. Whichever field remains effective at the lower levels has a very flat dose-response curve.

These experiments were designed to examine many parameters at once in order to enable us to outline the way different field conditions affect Physarum. As such, they probably raise more questions than they answer. For example, the separate roles of electric and magnetic fields deserve closer scrutiny. The individual effects of electric and magnetic fields were studied at a single intensity. The surprising finding that the E-field and B-field each had almost the same effect on Physarum may be a fortuitous result or it may mean that the same mechanism of interaction is responsible for the effects of both types of fields. Although we find it difficult to imagine a mechanism that responds to electric and magnetic fields in identical ways, the absence of a typical dose-response relationship with field intensities is further indication that an unconventional mechanism of interaction may be at work.

Part of the motivation for this study was the question of whether or not frequency modulated ELF fields have the same effect as cw ELF fields. Although the results are not unequivocal, we conclude that modulation of the type used here does not significantly alter the way magnetic and electric fields interact with biological systems. This is an important result which, if extended to other modes of frequency modulation, means that studies using cw fields have wide applicability. It may also provide insight into the nature of the mechanism of interaction between living organisms and extremely low frequency electromagnetic fields.

REFERENCES

1. A. S. Presman, Electromagnetic Fields and Life. Plenum Press, New York, 1970.

2. A. R. Liboff and R. A. Rinaldi, (Eds.), Electrically Mediated Growth Mechanisms in Living Systems, Ann. New York Acad. Sci. ,238, New York, 1974.

3. J. G. Llaurado, A. Sances, and J. H. Battocletti, (Eds.), Biological and Clinical Effects of Low-Frequency Magnetic and Electric Fields. Charles C. Thomas, Springfield, Illinois, 1974.

4. M. A. Persinger, ELF and VLF Electromagnetic Field Effects. Plenum Press, New York, 1974.

5. W. E. Southern, Orientation of Gull chicks exposed to project Sanguine's electromagnetic field. Science, 189, 143-145 (1975).

6. S. M. Bawin, and W. R. Adey, Sensitivity of calcium binding in cerebral tissue to weak environmental electric fields oscillating at low frequency. Proc. Natl. Acad. Sci. U.S.A. 73, 1999-2003 (1976).

7. R. P. Larkin, and P. J. Sutherland. Migrating birds respond to project Seafarer's electromagnetic field. Science 195, 777-779 (1977).

8. F. R. Moore, Geomagnetic disturbance and the orientation of nocturnally migrating birds, Science, 196, 682-683 (1977).

9. G. D. Antimonii, V. I. Badikov, A. A. Kel, E. A. Krasnov, and S. K. Sudakov, Effect of a modulated electromagnetic field on self-stimulation response in rats. Bull. Exp. Biol. Med. 82, 1451-1454 (1977).

10. M. T. Marron, E. M. Goodman, and B. Greenebaum, Mitotic delay in Physarum polycephalum induced by low intensity 60 Hz and 75 Hz electromagnetic fields. Nature (London) 254, 66-67 (1975).

11. E. M. Goodman, B. Greenebaum, and M. T. Marron, Effects of extremely low frequency electromagnetic fields on Physarum polycephalum.

Radiat. Res. 66, 531-540 (1976).

12. B. Greenebaum, E. M. Goodman, and M. T. Marron, Long-term effects of weak 45-75 Hz electromagnetic fields on the slime mold Physarum polycephalum. In Biological Effects of Electromagnetic Fields (C. C. Johnson, and M. L. Shore, Eds.), pp. 445-459, U.S. Department of Health, Education and Welfare Publication, 1976 (FDA) 77-8010 1.

13. J. W. Daniel, and H. H. Baldwin, Methods of culture for plasmodial myxomycetes. Methods Cell. Physiol. 1, 9-41, (1964).

14. B. Greenebaum, M. T. Marron, and E. M. Goodman, Method for ensuring comparable temperature in biological experiments using multiple incubators. Rev. Sci. Instrum. 48, 937-938 (1977).

15. C. Mittermayer, R. Braun, and H. P. Rusch, The effect of Actinomycin D on the timing of mitosis in Physarum polycephalum. Exp. Cell. Res. 38, 33-41 (1965).

16. E. Guttes, S. Guttes, and H. P. Rusch, Morphological observations and differentiation on Physarum polycephalum grown in pure culture. Devel. Biol. 3, 588-614 (1961).

17. Calibration of respirometers. In Manometric and Biochemical Techniques, (W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Eds.) 5th Ed., pp. 48-63. Burgess Publishing Co., Minneapolis, Minn., 1972.

18. R. B. Aronson, and D. D. Van Slyke. Manometric determination of CO_2 combined with scintillation counting of C-14. Anal. Biochem. 41, 173-188 (1971).

19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol. reagent. J. Biol. Chem. 193, 265-275 (1951).

20. M. Hollander and D. A. Wolfe, Nonparametric Statistical Methods, pp. 67-74. J. Wiley and Sons, New York, 1973.

21. M. T. Marron, E. M. Goodman, and B. Greenebaum, Effects of Weak Electromagnetic Fields on Physarum polycephalum: Mitotic Delay in Heterokaryons and Decreased Respirations. Experientia 34, 588-590 (1978).

22. E. S. Pearson and H. O. Hartley, (Eds.), Biometrika Tables for Statisticians. Table 10, p. 141, Biometrika Trust, University College, London, 1966.

Table 1. Notation for Describing Field Conditions.

1. E stands for an electric field and B stands for a magnetic field. The intensity of the field is indicated by the presence or absence of one or more tildes above the letter according to the following scheme:

E = 0.7 V/m	B = 2.0 G
$\tilde{E}$ = 0.14 V/m	$\tilde{B}$ = 0.4 G
$\tilde{\tilde{E}}$ = 0.035 V/m	$\tilde{\tilde{B}}$ = 0.1 G

2. The symbol E + B means that electric and magnetic fields were applied simultaneously in phase and at right angles to one another. Application of a single field is indicated by the use of only one symbol. The word "only" is often appended to the symbol to emphasize this situation.

3. The letters MOD appearing before a symbol such as MOD(E+B) mean that a modulated waveform was applied. The form of modulation was the kind referred to as "minimum-shift keying" in which a constant amplitude sinusoidal wave abruptly changes frequency at the peaks of the waveform thereby avoiding any discontinuity in the waveform. In these experiments shifts between 72 and 80 Hz were made at random half-cycles with an average of eight shifts per second. If MOD does not appear before a symbol, the fields were sinewave fields at 75 Hz.

Table 2. Means and standard deviations for mitotic cycle length and oxygen uptake distributions. Data is given in Figs. 2-9; the notation used to specify field condition is explained in Table 1.

FIELD CONDITION	ΔMITOTIC CYCLE LENGTH		ΔOXYGEN UPTAKE	
	MEAN (hr)	STD. DEV. (hr)	MEAN (%)	STD. DEV. (%)
E+B	0.64	0.49	-15.7	11.0
B	0.46	0.36	- 8.1	6.4
E	0.39	0.27	- 9.1	5.1
$\tilde{E}+\tilde{B}$	0.42	0.26	- 4.2	2.5
MOD(E+B)	0.55	0.53	- 7.0	12.2
MOD($\tilde{E}+\tilde{B}$)	0.40	0.34	- 3.7	7.1
MOD($\tilde{\tilde{E}}+\tilde{\tilde{B}}$)	0.43	0.33	- 3.1	3.7

FIGURE CAPTIONS

Figure 1. Average length of intermitotic cycle measured from the time nutrient medium is added to a fused macroplasmodium to the second metaphase configuration of the nuclei. Each point represents the average of five to ten observations on separate cultures. The circles are data taken from control cultures and the squares are data taken from cultures exposed to 75 Hz 2.0 G, 0.7 V/m. The standard error for each point is in the range 0.03 to 0.10 hrs and is too samll to be seen as an error bar on this scale.

Figure 2. Distribution of differences in the length of the mitotic cycle for exposed cultures taken relative to average daily control culture cycle based on data in Fig. 1. The average control value for the appropriate day has been subtracted from each observation made on exposed cultures. N is the total number of observations on exposed cultures represented in the histogram. Field exposure conditions and histogram statistics are shown in the Figure.

Figure 3. Distribution of differences in the length of the mitotic cycle for cultures exposed to 2.0 G magnetic fields.

Figure 4. Distribution of differences in the length of the mitotic cycle for cultures exposed to 0.7 V/m electric fields.

Figure 5. Distribution of differences in the length of the mitotic cycle for cultures exposed to E and B fields of 0.14 V/m and 0.4 G.

Figure 6. Distribution of differences in the length of the mitotic cycle for cultures exposed to modulated E and B fields of 0.7 V/m and 2.0 G.

Figure 7. Distribution of differences in the length of the mitotic cycle for cultures exposed to modulated E and B fields of 0.14 V/m and 0.4 G.

Figure 8. Distribution of differences in the length of the mitotic cycle for cultures exposed to modulated E and B fields of 0.035 V/m and 0.1 G.

Figure 9. Respiration rates for exposed microplasmodial cultures relative to average daily control culture respiration rates. Histogram bins are 5% wide; the center value of the bin is given below on the abcissa. Each point represents a set of rate measurements performed upon a single, exposed culture taken relative to the average of rate measurements in two control cultures determined at the same time. Data given on the right are average values $\pm$ standard errors computed from the raw data. The probability that the distribution mean differs from zero only by chance is also listed; these probabilities are derived using the Wilcoxon Signed Rank statistic.

Figure 10. Statistical comparison of distributions to test reproducibility and internal consistency of the data. The solid bar gives the results for the Wilcoxon Signed Ranks test; the open bar gives the t-test statistic. The $p = 0.01$ and $p = 0.001$ levels for large samples are shown on the graph. The symbols E, B, $\tilde{E}$, etc. are defined in Table 1.

a. Test for data reproducibility by comparing distributions of cycle length data for two sets of cultues each exposed to the same field conditions but at different times. For example, cultures in set B_1 were exposed to a 75 Hz magnetic field of 2.0 G; cultures in set B_2 were also exposed to a 75 Hz magnetic field of 2.0 G but these experiments began almost one year later. N1/N2 is the number of data in the first/second set.

b. Test of internal consistency by comparison of equivalent distributions for different field conditions. There are three sets of four comparisons all of which display internal consistency. Note also that the E-distributions, the B-distributions, and the $(\tilde{E}+\tilde{B})$-distributions are all statistically equivalent.

Figure 11. Comparison of mitotic cycle distributions for different field conditions. Data are pooled so that all data taken at a particular set of field conditions are treated as a single group. The solid bar gives the results for the Wilcoxon Signed Rank test; the open bar gives the t-test

statistic. The p = 0.01 and p = 0.001 levels for large samples are shown on the graph as vertical lines. The symbols E, B, $\tilde{E}$, etc. are defined in Table 1. Entries in the column headed by N1/N2 are the number of data in the first/second distribution.

a. Conclusions: E+B ≠ E-only or B-only. Simultaneous application of electric and magnetic fields produces a different mitotic cycle lengthening distribution than that observed when fields are applied individually. At these levels (2.0 G and 0.7 V/m) the individual fields produce statistically identical results.

b. Conclusions: $\tilde{E}+\tilde{B}$ = E-only and B-only; $\tilde{E}+\tilde{B} \neq$ E+B. Electric and magnetic fields applied at a level five times weaker cause a different result to occur. These weaker fields produce the same effect as either more intense E-fields alone or more intense B-fields alone. This finding agrees with those of Figure 10.

c. Conclusions: MOD(E+B) ≠ MOD($\tilde{E}+\tilde{B}$); MOD(E+B) = MOD($\tilde{\tilde{E}}+\tilde{\tilde{B}}$); MOD($\tilde{E}+\tilde{B}$) = MOD($\tilde{\tilde{E}}+\tilde{\tilde{B}}$). Reduction of field intensity by five times causes the mitotic cycle lengthening distribution to change. A further decrease in intensity by four times has no effect.

d. Conclusions: MOD(E+B) = $\tilde{E}+\tilde{B}$ and B-only; MOD(E+B) ≠ E+B or E. Modulation of the fields results in a mitotic cycle lengthening distribution similar to unmodulated fields of weaker intensity. Unlike the unmodulated fields, the MOD(E+B) results are similar to the B-only exposures.

e. Conclusions: MOD($\tilde{E}+\tilde{B}$) ≠ E+B; MOD($\tilde{E}+\tilde{B}$) = $\tilde{E}+\tilde{B}$; MOD($\tilde{E}+\tilde{B}$) = B-only or E-only. Modulated fields at 0.14 V/m and 0.4 G produce results statistically identical to unmodulated fields of the same intensity. Like the unmodulated fields, MOD($\tilde{E}+\tilde{B}$) data are indistinguishable from those produced by more

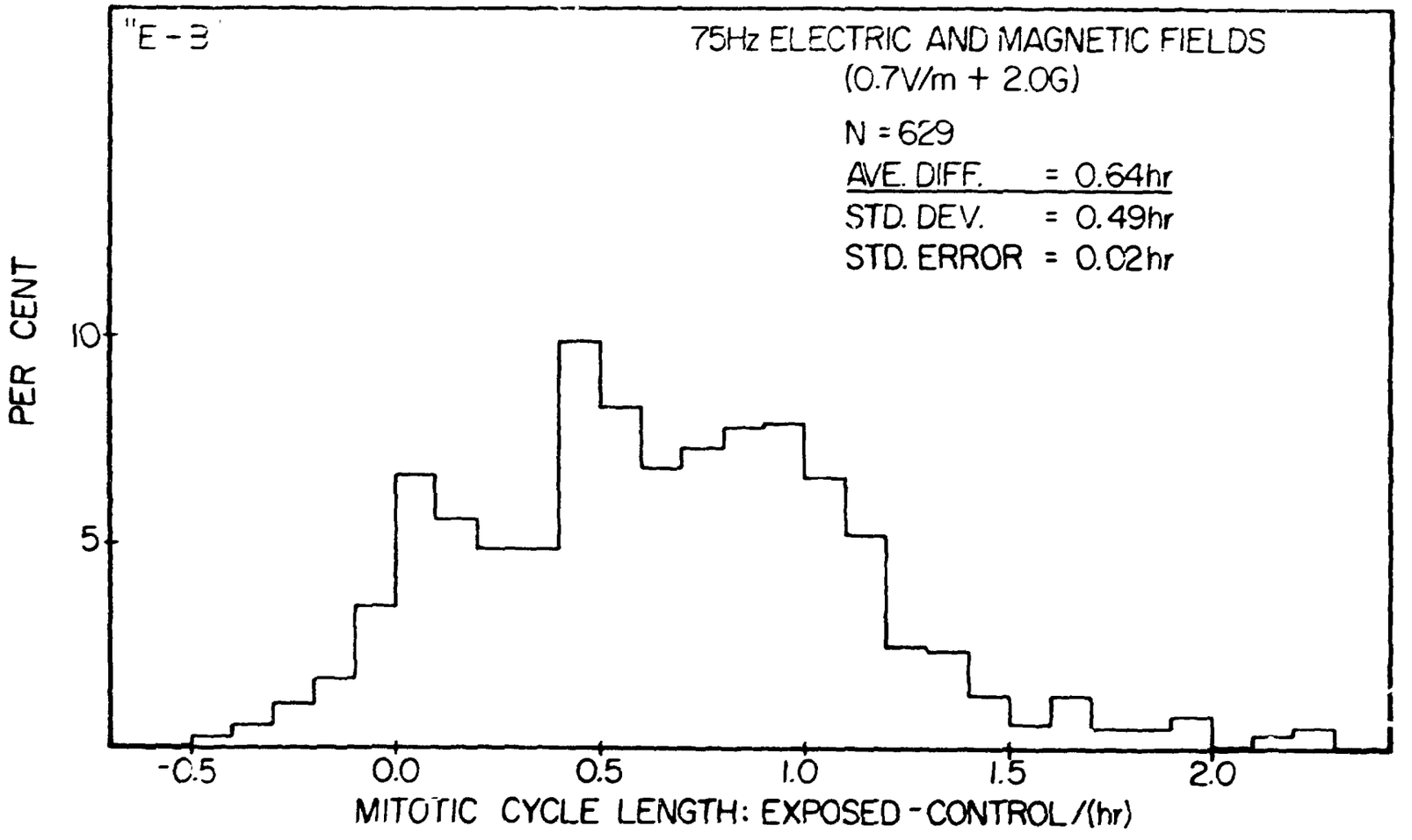
intense unmodulated E-only and B-only fields.

f. Conclusions: $MOD(\tilde{\tilde{E}}+\tilde{\tilde{B}}) \neq E+B$; $MOD(\tilde{\tilde{E}}+\tilde{\tilde{B}}) = \tilde{E}+\tilde{B}$; $MOD(\tilde{\tilde{E}}+\tilde{\tilde{B}})$ = B-only or E-only. Decreasing field intensities further to 0.035 V/m and 0.1 G has no apparent effect on the mitotic cycle lengthening distribution.

Figure 12. Visual summary of the statistical analysis presented in Figure 11.

FIG. 1

FIG. 2


"E-3
75Hz ELECTRIC AND MAGNETIC FIELDS
(0.7V/m + 2.0G)
N = 629
AVE. DIFF. = 0.64hr
STD. DEV. = 0.49hr
STD. ERROR = 0.02hr
PER CENT
10
5
-0.5
0.0
0.5
1.0
1.5
2.0
MITOTIC CYCLE LENGTH: EXPOSED - CONTROL /(hr)

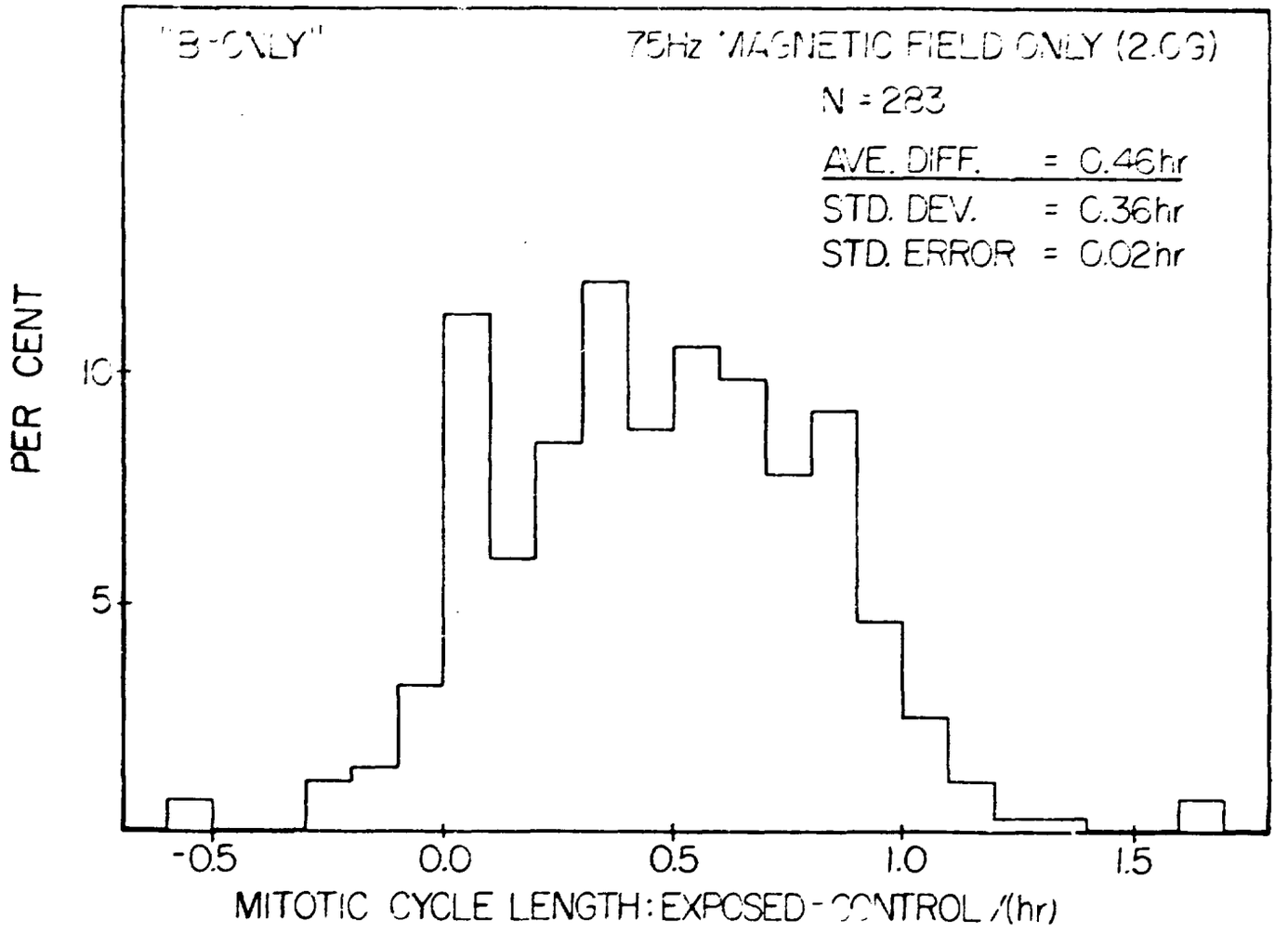

"B-ONLY"
75Hz MAGNETIC FIELD ONLY (2.0G)
N = 283
AVE. DIFF. = 0.46hr
STD. DEV. = 0.36hr
STD. ERROR = 0.02hr
PER CENT
10
5
-0.5
0.0
0.5
1.0
1.5
MITOTIC CYCLE LENGTH: EXPOSED - CONTROL (hr)

FIG. 3

PER CENT
5
0
5
E ONLY
75Hz ELECTRIC FIELD ONLY 15.7 V/m
N = 205
AVE. DIFF. = 0.39hr
STD. DEV. = 0.27hr
STD. ERROR = 0.02hr

FIG. 4

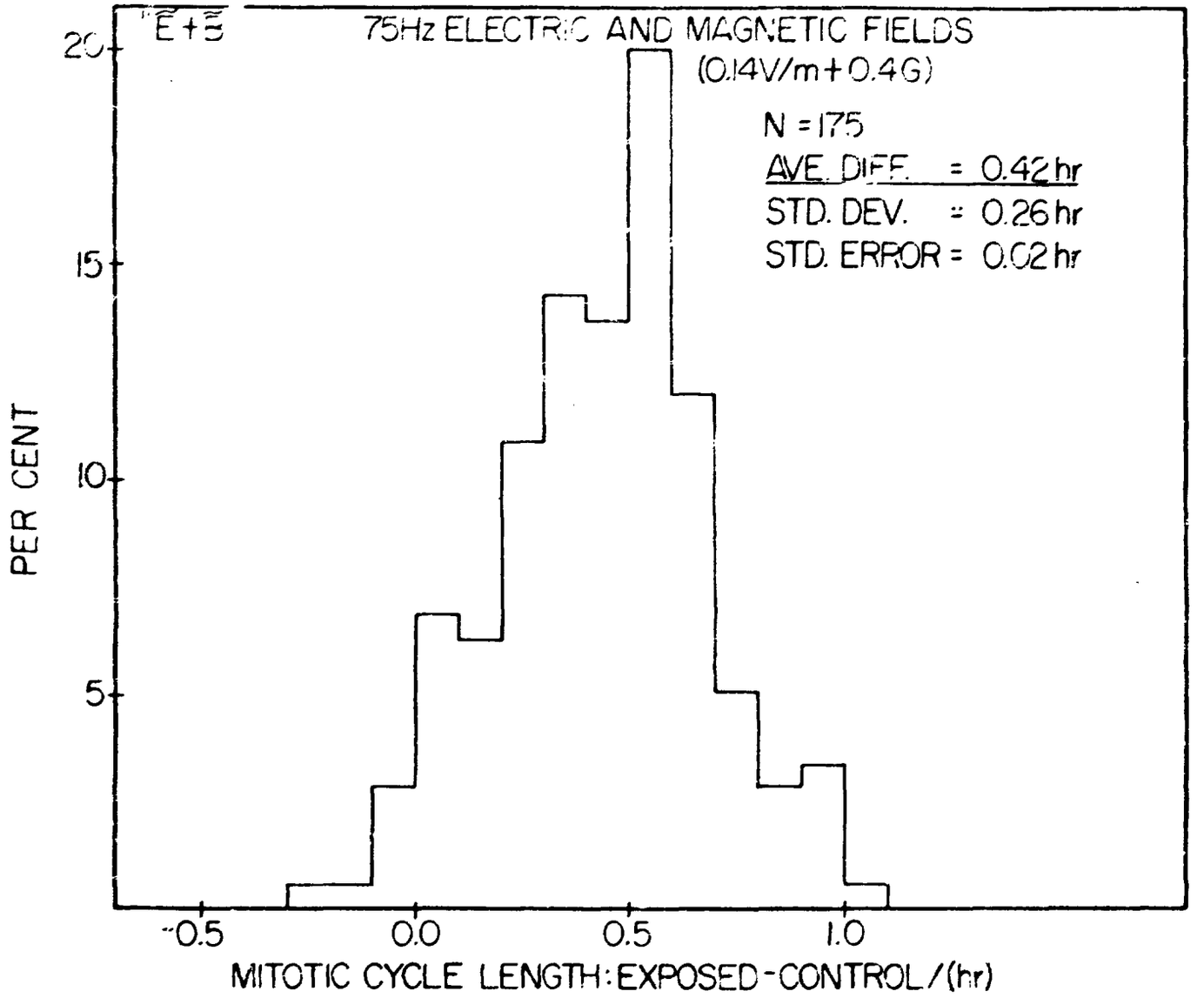
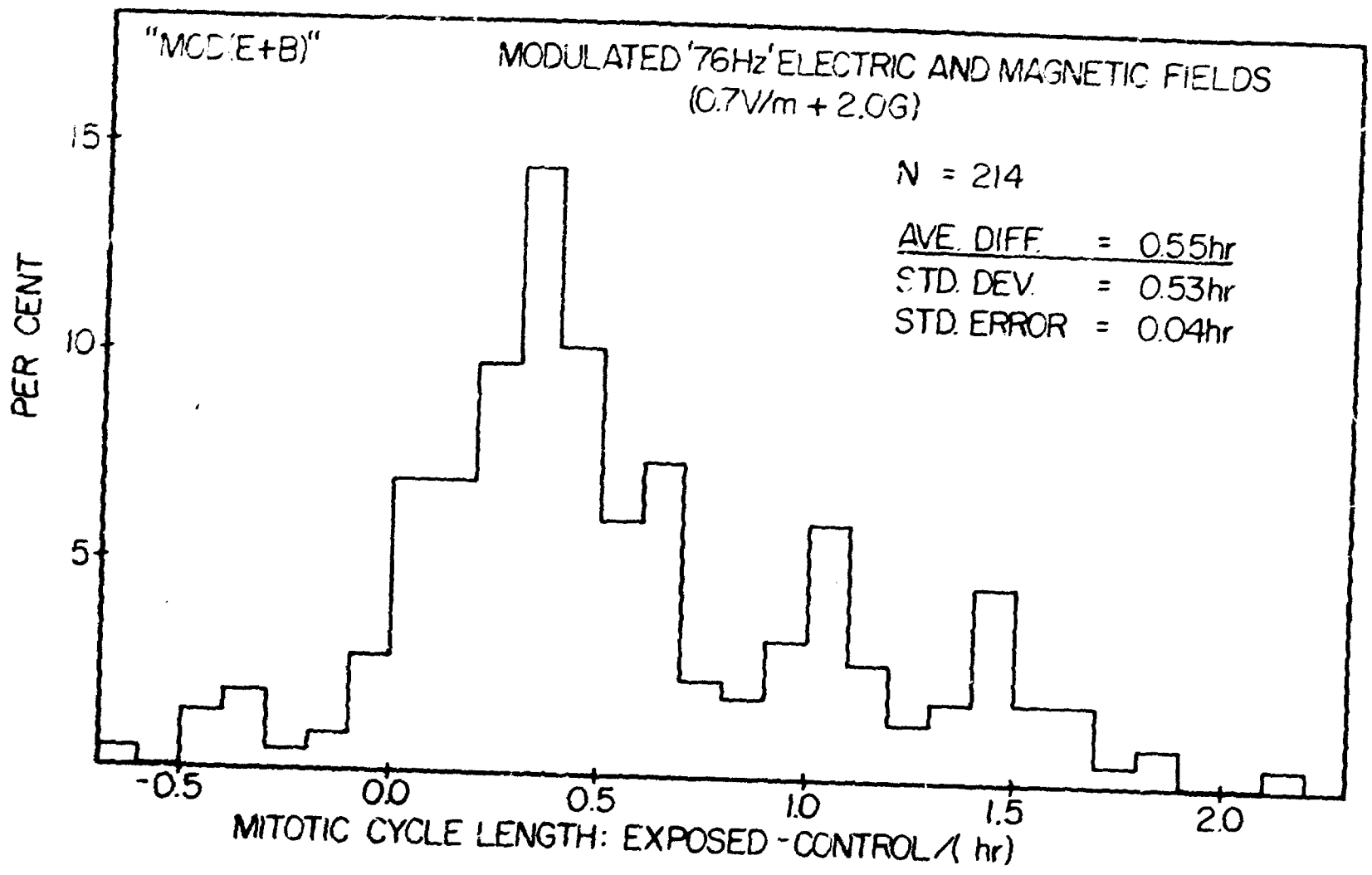

$\vec{E}+\vec{B}$
75Hz ELECTRIC AND MAGNETIC FIELDS
(0.14V/m+0.4G)
N = 175
AVE. DIFF. = 0.42 hr
STD. DEV. = 0.26 hr
STD. ERROR = 0.02 hr
PER CENT
20
15
10
5
-0.5
0.0
0.5
1.0
MITOTIC CYCLE LENGTH: EXPOSED - CONTROL / (hr)

FIG. 5

FIG. 6


"MOD (E+B)"
MODULATED 76Hz ELECTRIC AND MAGNETIC FIELDS
(0.7V/m + 2.0G)
N = 214
AVE. DIFF. = 0.55hr
STD. DEV. = 0.53hr
STD. ERROR = 0.04hr
PER CENT
15
10
5
-0.5
0.0
0.5
1.0
1.5
2.0
MITOTIC CYCLE LENGTH: EXPOSED - CONTROL (hr)

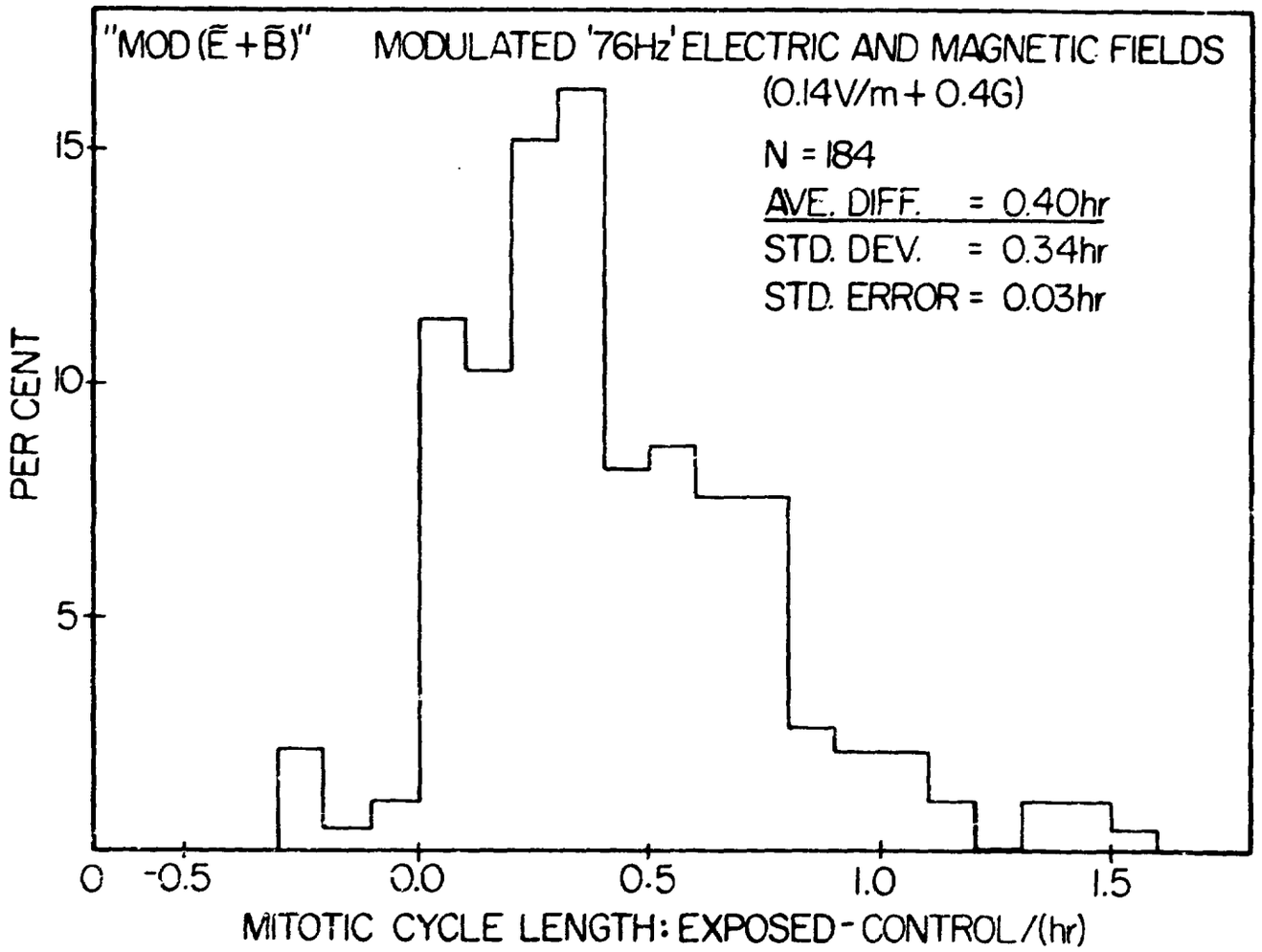
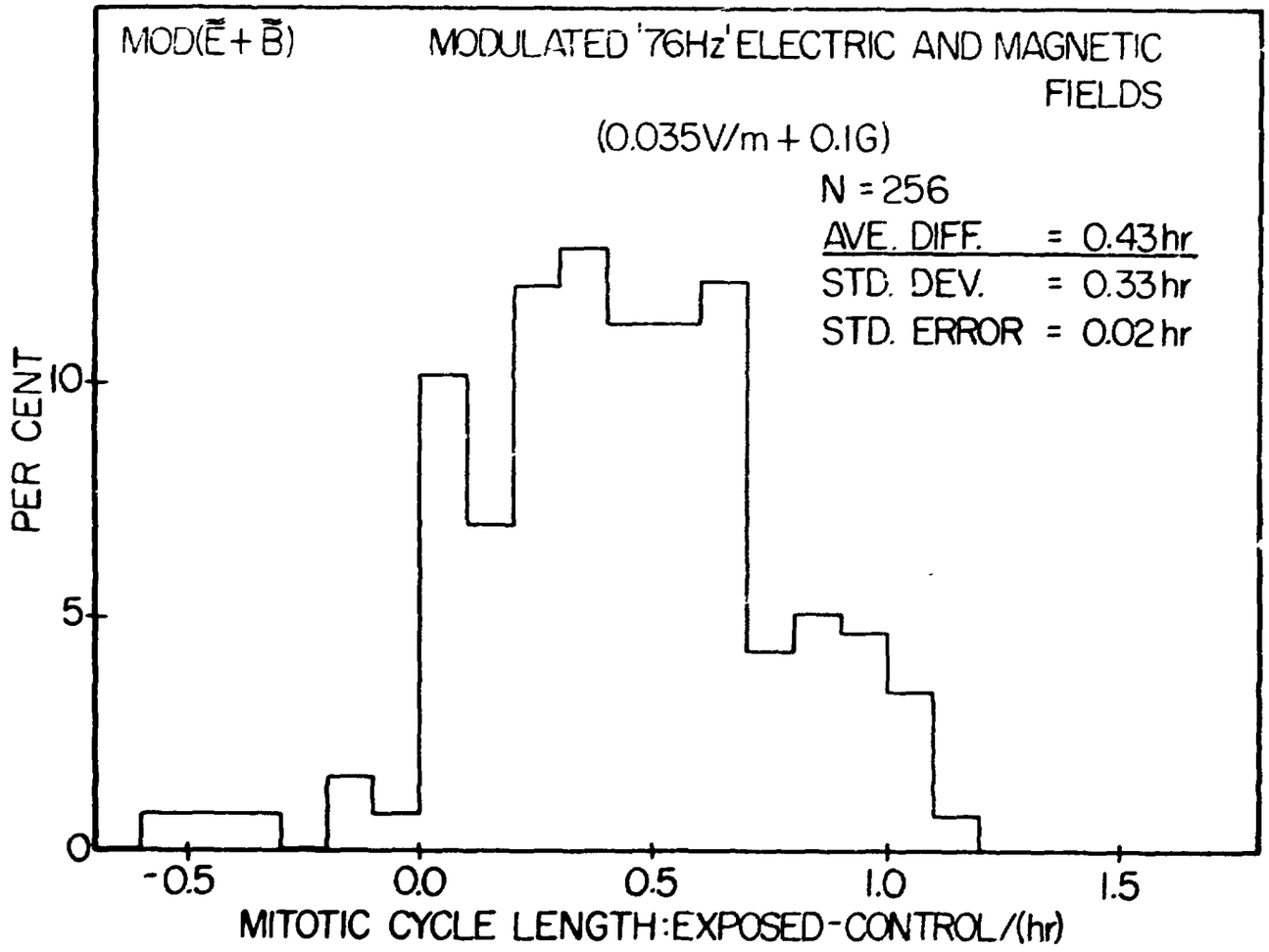

"MOD (Ẽ + B̃)" MODULATED '76Hz' ELECTRIC AND MAGNETIC FIELDS
(0.14V/m + 0.4G)
N = 184
AVE. DIFF. = 0.40hr
STD. DEV. = 0.34hr
STD. ERROR = 0.03hr
PER CENT
15
10
5
0
-0.5
0.0
0.5
1.0
1.5
MITOTIC CYCLE LENGTH: EXPOSED - CONTROL/(hr)

FIG. 7

FIG. 8


MOD($\vec{E}$ + $\vec{B}$)
MODULATED '76Hz' ELECTRIC AND MAGNETIC FIELDS
(0.035V/m + 0.1G)
N = 256
AVE. DIFF. = 0.43hr
STD. DEV. = 0.33hr
STD. ERROR = 0.02hr
PER CENT
10
5
0
-0.5
0.0
0.5
1.0
1.5
MITOTIC CYCLE LENGTH: EXPOSED - CONTROL/(hr)

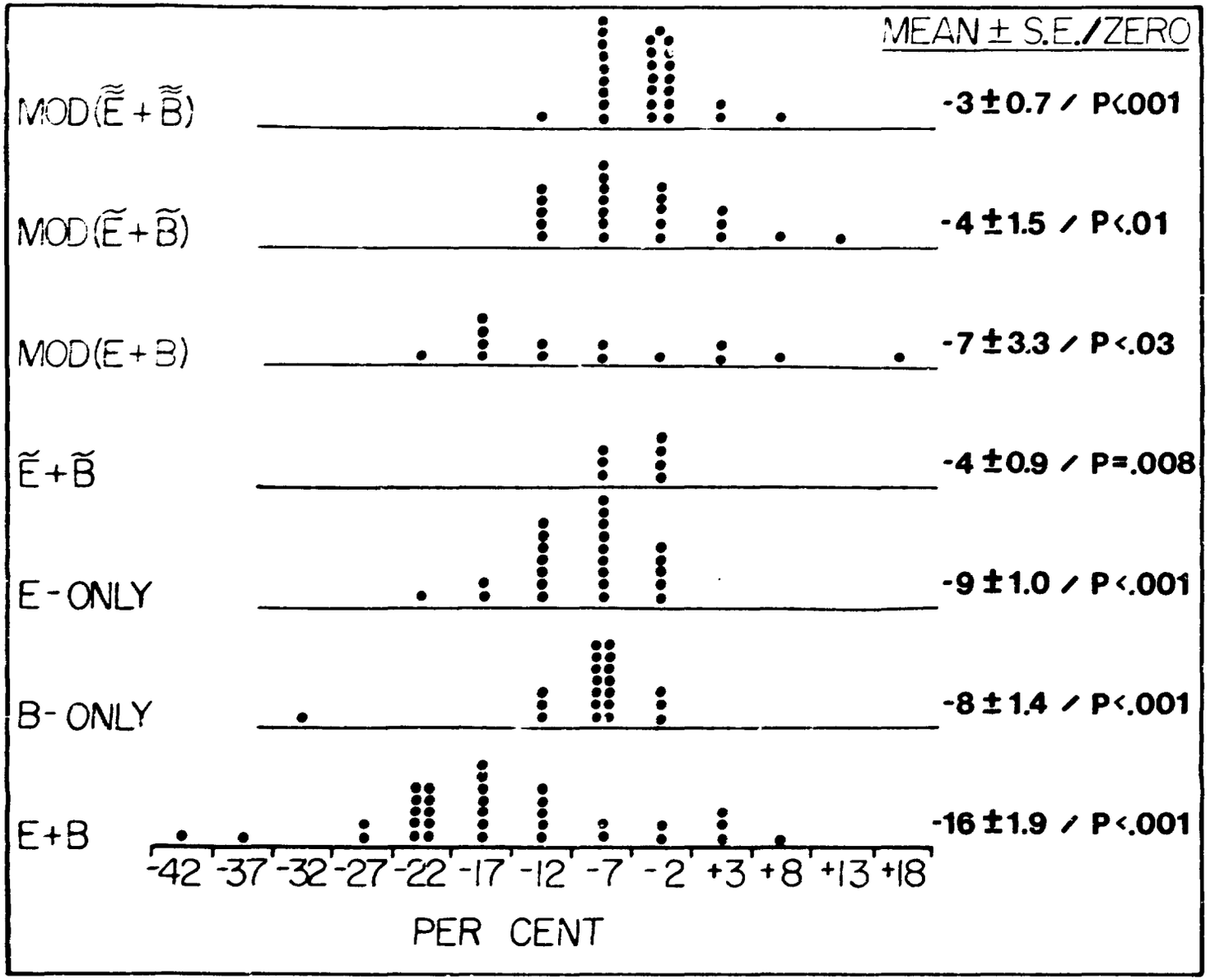
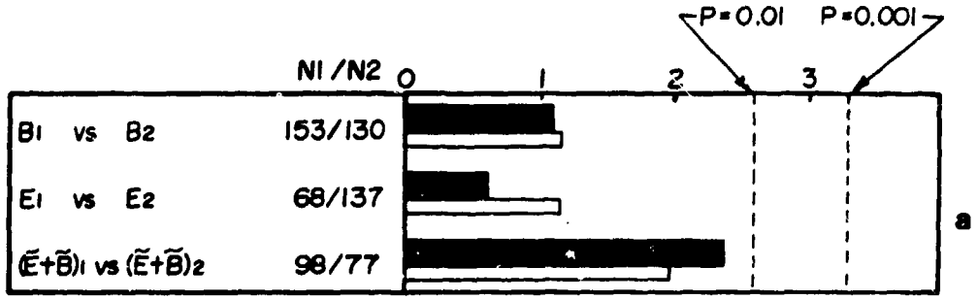

MEAN ± S.E./ZERO
MOD($\tilde{\tilde{E}}$ + $\tilde{\tilde{B}}$)
-3 ± 0.7 / P<.001
MOD($\tilde{E}$ + $\tilde{B}$)
-4 ± 1.5 / P<.01
MOD(E + B)
-7 ± 3.3 / P<.03
$\tilde{E}$ + $\tilde{B}$
-4 ± 0.9 / P=.008
E - ONLY
-9 ± 1.0 / P<.001
B - ONLY
-8 ± 1.4 / P<.001
E + B
-16 ± 1.9 / P<.001
-42 -37 -32 -27 -22 -17 -12 -7 -2 +3 +8 +13 +18
PER CENT

FIG. 9


P=0.01
P=0.001
N1/N2
0
1
2
3
B1 vs B2
153/130
E1 vs E2
68/137
(Ẽ+B̃)1 vs (Ẽ+B̃)2
98/77
a

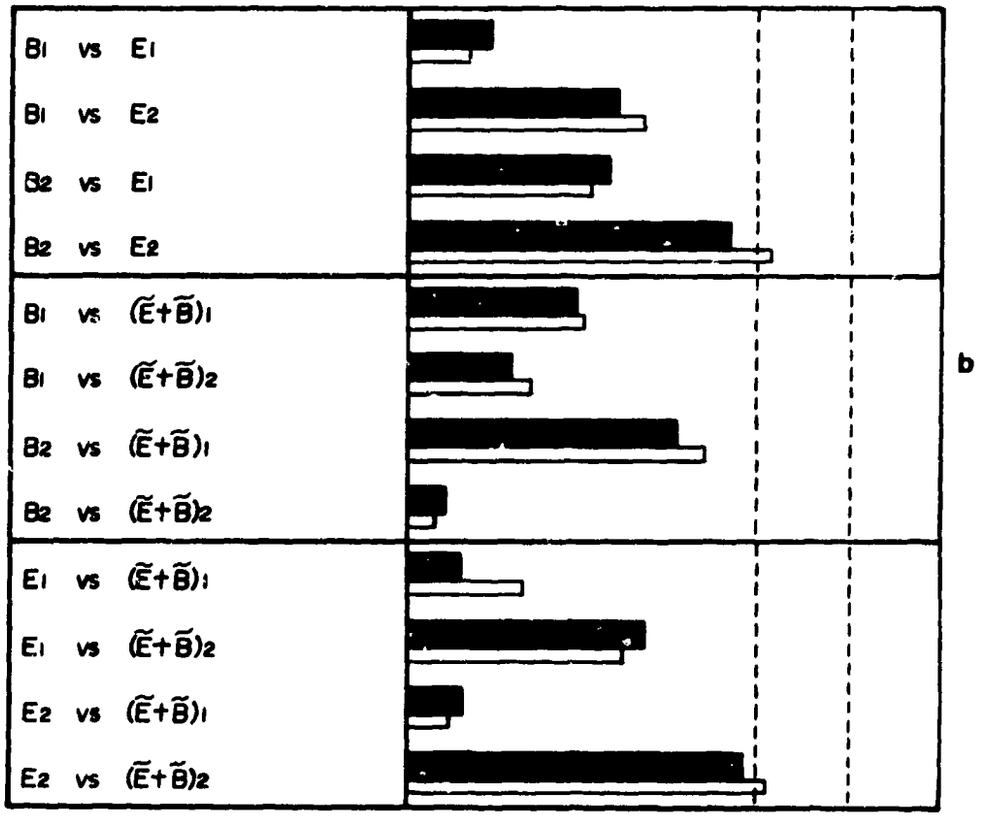

B1 vs E1
B1 vs E2
B2 vs E1
B2 vs E2
B1 vs (Ẽ+B̃)1
B1 vs (Ẽ+B̃)2
B2 vs (Ẽ+B̃)1
B2 vs (Ẽ+B̃)2
E1 vs (Ẽ+B̃)1
E1 vs (Ẽ+B̃)2
E2 vs (Ẽ+B̃)1
E2 vs (Ẽ+B̃)2
b

FIG. 10

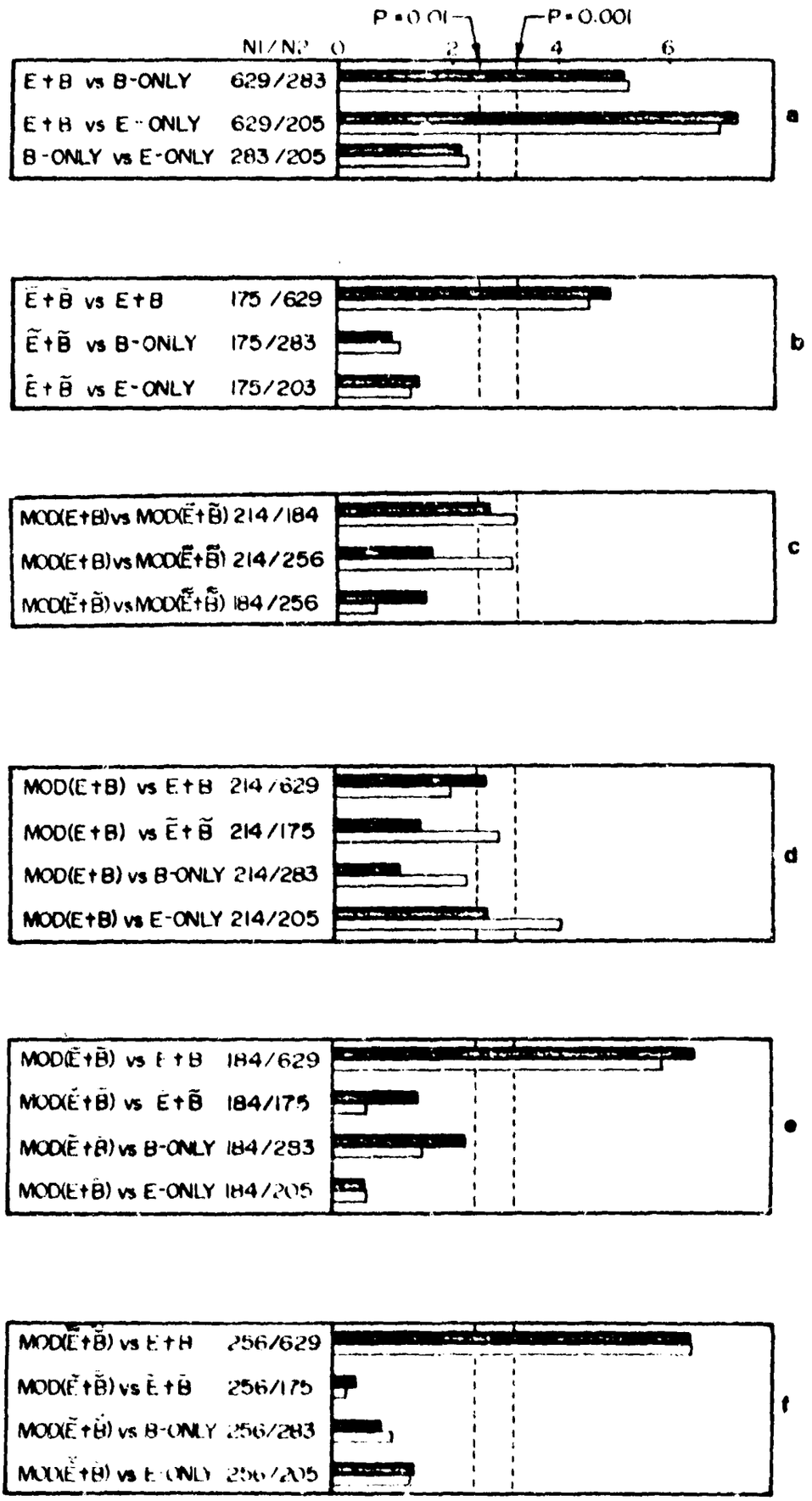

P = 0.01
P = 0.001
N1/N2
0
2
4
6
E+B vs B-ONLY 629/283
E+B vs E-ONLY 629/205
B-ONLY vs E-ONLY 283/205
a
Ẽ+B vs E+B 175/629
Ẽ+B̃ vs B-ONLY 175/283
Ê+B̃ vs E-ONLY 175/203
b
MOD(E+B)vs MOD(Ẽ+B̃) 214/184
MOD(E+B)vs MOD(Ẽ+B̃) 214/256
MOD(Ẽ+B̃)vs MOD(Ẽ+B̃) 184/256
c
MOD(E+B) vs E+B 214/629
MOD(E+B) vs Ẽ+B̃ 214/175
MOD(E+B) vs B-ONLY 214/283
MOD(E+B) vs E-ONLY 214/205
d
MOD(Ẽ+B̃) vs E+B 184/629
MOD(Ẽ+B̃) vs Ẽ+B̃ 184/175
MOD(Ẽ+B̃) vs B-ONLY 184/283
MOD(Ẽ+B̃) vs E-ONLY 184/205
e
MOD(Ẽ+B̃) vs E+B 256/629
MOD(Ẽ+B̃) vs Ẽ+B̃ 256/175
MOD(Ẽ+B̃) vs B-ONLY 256/283
MOD(Ẽ+B̃) vs E-ONLY 256/205
f

FIG. 11

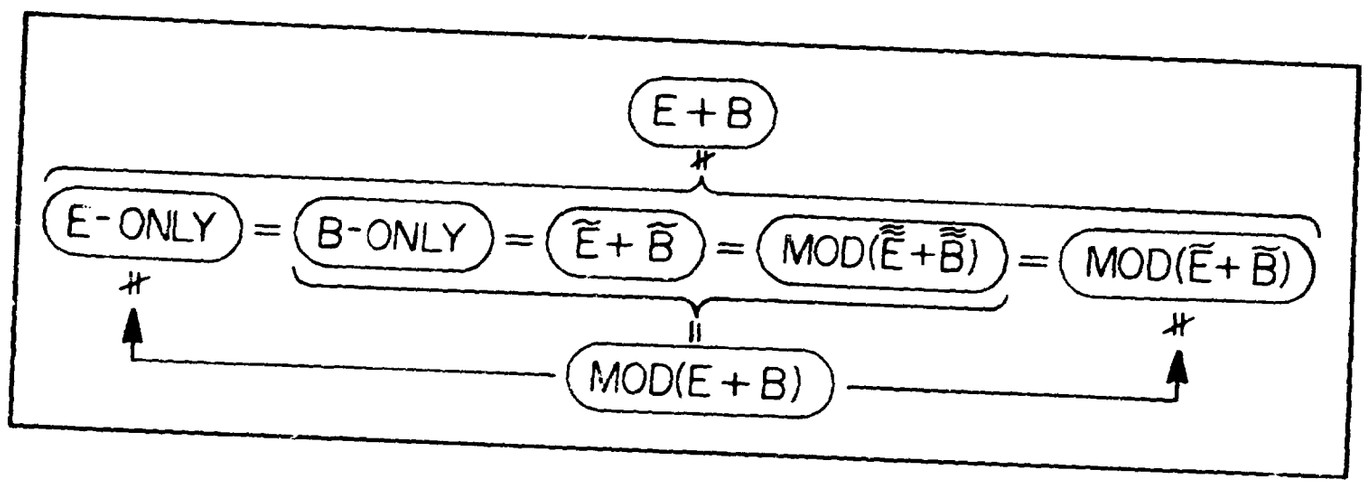

E+B
E-ONLY
=
B-ONLY
=
$\widetilde{E}+\widetilde{B}$
=
MOD($\widetilde{\widetilde{E}}+\widetilde{\widetilde{B}}$)
=
MOD($\widetilde{E}+\widetilde{B}$)
MOD(E+B)

FIG. 12

Effects of Extremely Low Frequency Electromagnetic Fields on *Physarum polycephalum*: Observations on Ploidy, Conditioning and External Factors

SUMMARY

Physarum polycephalum cultures exhibiting a lengthened mitotic cycle and depressed respiration rate after extended exposure to 75Hz, 2.0G and 0.7 V/m, electromagnetic fields (EMF) are examined for detectable changes in chromosome numbers. Since *Physarum* is polyploid, only a distribution of chromosome numbers can be discussed. We see no evidence of a divergence between exposed and control cultures; any undetected difference in chromosome numbers is less than 6% ($p<0.05$). When cultures exhibiting the altered physiological parameters are placed in a control environment, latent effects of exposure remain for an extended period. Results of additional experiments are presented that are designed to rule out certain non-EMF factors as being responsible for the biological changes. Factors tested include ambient electromagnetic fields, incubator differences, electrolytic effects, and investigator bias.

1.

INTRODUCTION

We have previously reported that weak electromagnetic fields (EMF) of the same general frequency and strength as those found in an industrialized environment cause changes in the myxomycete Physarum polycephalum. In particular, we have shown that exposure of the slime mold to a variety of EMF conditions produces a lengthening of the mitotic cell cycle, a depression of the respiration rate, and a retardation of the spontaneous protoplasmic streaming (Marron et al. (1975), Goodman et al. (1976), and Goodman et al. (1979)). This report presents evidence that no major change has occurred in the ploidy of our cultures. Goodman et al. (1976) have also reported that placing exposed cultures that exhibit a lengthened mitotic cycle into a control environment results in a slow return of the cycle length to control levels. We present evidence here that these cultures still preserve some latent effects of exposure. Finally, we report results of experiments that tend to rule out a number of non-EMF factors as possible causes of the observed physiological changes in Physarum. These factors are incubator differences, ambient electromagnetic fields, electrolysis of the medium, and investigator bias.

EXPERIMENTAL METHODS

Culture and Exposure Techniques

Our culture techniques, experimental methods, and exposure apparatus have been described in detail by Goodman et al. (1976) and Greenebaum et al. (1977a). Briefly, cultures of submerged Physarum microplasmodia (strain M_3C VII(IIe)) are grown in rectangular flasks, shaken for aeration; electrodes forming opposite sides of the flasks set up the electric fields. Magnetic field coils surround the flasks. The entire apparatus is enclosed

in an incubator which is maintained at 25.5 + 0.3°C. Temperature controls of all incubators are interconnected to minimize possible temperature differences (Groenebaum et al. (1977b)). Incubators are similar in all respects, except that the magnetic field coils and electric field electrodes are not energized in the control incubator.

The submerged shake cultures of microplasmodia maintained in the non-energized control incubator serve as control cultures and as a source of microplasmodia for experimental purposes. At the start of a series of experiments to determine the effects of a particular EMF environment, microplasmodia derived from control cultures are used as inoculum for cultures introduced into the appropriate fields. All experiments were duplicated by introducing a second set of fresh cultures into the field two to eight weeks after initial exposure of the first set. All cultures in both the control and EMF environments were routinely sub-cultured into fresh nutrient medium on a rigid 48 hour/48 hour/72 hour schedule. To ensure that microplasmodia used in the mitotic cycle length and respiration measurements were growing in undepleted medium, that is, in their so-called logarithmic growth phase, inocula from control and exposed stock cultures were placed in new growth medium 24 hours prior to making any measurements. Microplasmodia from these fresh cultures were then used in setting up division cycle or respiration experiments.

Nuclear Division Cycle and Respiration Rate Measurements

Mitotic cycle length is measured by placing washed microplasmodia on filter paper, supported in a Petri dish containing nutrient medium by an absorbent cotton pad, as described by Goodman et al. (1976, 1979). Experiments usually consist of 10 plates containing microplasmodia grown in the

control environment and 10 plates containing microplasmodia from cultures grown in EMF. Within an hour, the microplasmodia coalesce into a single, large macroplasmodium, and nutrient medium is added at this time. Mitotic cycle lengths are characterized by the time required for each macroplasmodium to reach the second metaphase configuration after the addition of medium. All nuclei in a single macroplasmodium undergo mitosis in virtual synchrony. The stages of the mitotic cycle are determined by observing ethanol-fixed smears with a phase-contrast microscope.

Methods for measuring respiration rates have been described by Goodman and Beck (1974). Duplicate 0.5 ml samples of control and exposed microplasmodia are placed in sterile, calibrated Warburg reaction vessels. Growth medium (2.5 ml) is added and the flasks are equilibrated at 25.0 $\pm$ 0.1°C. The CO_2 evolved is trapped in the center well of the flask using filter paper saturated with hyamine hydroxide (Aronson and Van Slyke, 1971). At the conclusion of the experiments, microplasmodia are removed from the vessel, and their pigment is extracted in a trichloroacetic acid-acetone-water mixture. Protein is estimated using the colorimetric procedure of Lowry et al. (1951), using bovine serum albumin as a standard.

CHROMOSOME NUMBERS

Measurements

Since Physarum is a polyploid organism, it is difficult to look for genetic changes in our cultures directly, however; a distribution in the number of chromosomes may be examined to see whether any gross change in nuclear material has occurred. The methods employed are based on the work of Mohberg et al. (1973), who established that the distribution of nuclear sizes in Physarum is closely related to the distribution of chromosome

numbers. Using the procedures of Mohberg and Rusch (1971), nuclei were isolated from cultures exposed to 45 Hz, 3.0G, 0.7 V/m EMF and from control cultures. The isolated nuclei were photographed under a phase-contrast microscope (250x) on an A-O Spencer phase-contrast hemocytometer. Photographs were enlarged (4x) and the distribution of nuclear diameters was measured in each photograph. Hemocytometer rulings, visible in each photograph, were checked to ensure consistency of all measurements and to establish unambiguously the degree of magnification. Typical photographs of exposed and control nuclei are shown in Figure 1.

Results

Measurements of exposed nuclei (N=104) yielded a mean diameter of 3.16 $\pm$ 0.05 micrometer (95% confidence limits); the mean diameter of control nuclei (N=213) was 3.14 $\pm$ 0.03 micrometer. These two means are not significantly different from each other. However, to report a negative test without indicating the sensitivity of the test is to make an incomplete report; one should set an upper limit on any potential undetectable differences. The power function of the t-test (Pearson and Hartley, 1976) may be used to estimate how large a difference between the means would have to be before it could be detected; this function estimates the sensitivity of a test at a predetermined confidence level based on the scatter in the data. The two distributions of nuclear diameters are such that we should be able to detect a difference as small as 0.09 micrometers between the means with 95% confidence; this difference corresponds to 3% of the mean values.

A more useful figure for comparison is the maximum difference in chromosome number. Using the measurements of Mohberg et al. (1973) we have found an empirical relationship between diameter and chromosome number. The best

fit between chromosome number and diameter is found for a linear, rather than a second- or third-power equation

MEAN CHROMOSOME NUMBER = 41.2 x (MEAN DIAMETER/micrometers) - 73.6,

although the fit for different powers of the mean diameter is very nearly the same, as can be seen from the correlation coefficients (Pearson product-moment correlation coefficient): 0.9831 (1st power), 0.9798 (2nd power), and 0.9706 (3rd power). Based on this relationship, we conclude that if a difference in chromosome number exists between exposed and control nuclei, it is less than 4 ($p<0.05$) out of a total of approximately 60 chromosomes.

Discussion

One might suggest that differences in respiration and mitosis between exposed and control cultures could be attributed to slow "genetic drift." These data tend to rule out that possibility. Additional evidence against the "slow drift" suggestion is presented by our earlier works (Goodman, et al, 1976, 1979). We have repeatedly placed into the EMF environments new cultures derived from our main control cultures. In all cases, the biological effects induced by exposure to a given EMF environment were consistent with those observed in cultures introduced into the same fields at other times. If "genetic drift" were affecting the parameters being measured, discernable differences between the various sets of cultures exposed to the same EMF environment would be observed, unless the drift itself is induced by the exposure. Our measurements of chromosome numbers are consistent with the argument that chromosomal changes in the cultures are not the mechanism through which the observed effects are produced. However, it is still possible that selection or changes at the level of the gene are occurring reproducibly under the influence of the applied fields.

As a side note, it is interesting that our observations indicate that there is little change in chromosome number after long-term culture in both exposed and control environments. These results are somewhat at variance with those of McCullough et al. (1973), who noted death of cultures of the CL and B173xB174 strains, grown on agar, after 85 and 235 days, respectively. About 20 days before death McCullough et al. observed gross nuclear enlargement. They state that the senescence and death of Physarum cultures grown for many months is a common phenomenon. However, both exposed and control cultures in our experiments have been maintained in submerged cultures for more than 1700 days and continue to exhibit a diameter typical of both our strain and those used by McCullough et al. (Mohberg et al. (1973)). We speculate that the differences in culture method (submerged cultures vs. agar) may account for the discrepancy between our experience and that of McCullough et al.

LATENT EFFECTS OF EXPOSURE

In a series of experiments designed to test the persistence of effects induced by exposure to EMF, cultures were continuously exposed to 75Hz, 2.0 G and 0.7 V/m, fields until they exhibited a lengthened mitotic cycle. They were then removed from the fields and placed in the control environment. Goodman et al. (1976) have reported that the observed changes were no longer detectable after about 30 days in the control environment. After about 60 days in one experiment and after about 300 days in another, the cultures were removed from the control environment and reintroduced into the EMF. The lengthened mitotic cycle reappeared in 20 to 30 days (Fig. 2), notably sooner than the 90-120 days originally required when a culture was initially exposed to EMF (Goodman et al. (1976)).

Some part of the organism apparently "remembers" its previous exposure. If one proceeds from our earlier finding that, while subtle "genetic" changes may have occurred, gross changes in ploidy have not, one tends to seek a mechanism that does not require a genetic change. Several investigators have suggested that EMF effects may be caused by structural changes in the membrane (see, for example, Bawin et al. (1978) and Grodsky (1976)); such a hypothesis is consistent with our findings.

TESTS OF ALTERNATIVE HYPOTHESES

Incubator and Extraneous Field Effects

To test alternate hypotheses for the origin of observed differences between exposed and control cultures we conducted several series of experiments. The first set of alternative hypotheses was that the effects were due to extraneous fields or other influences arising from either the location of the laboratory or differences in the incubators. We conducted two sets of experiments to test these influences. One set was conducted without an incubator in a laboratory room removed from the location of our other experiments; the blind experimental protocol discussed below was used in this set. The other set of experiments was conducted in a third location, using a single large incubator (Controlled Environments, Ltd., Model EY-13) that held both exposed and control cultures. At the start of each of these two sets of experiments, four stock cultures were derived from our main control cultures; two of the four were designated as controls and two as experimentals. Each pair was placed in a newly-constructed field-generating unit similar in design to our usual apparatus. The electric and magnetic circuitry was not energized in the control half of the unit. Experimental cultures were exposed to 45 Hz sinusoidal EMF, 3.0 G and 0.7 V/m; stray magnetic fields at the location of the control

cultures were 0.06 G.

In experiments using a remote, single incubator we observed a depression in the respiration rate of exposed cultures; the data are summarized in Table I. While respiration rate was the only parameter measured for experiments conducted in the single incubator, the experiments conducted without an incubator in the second remote room examined both respiration rate and mitotic cycle length. As shown in Table I, these exposed cultures displayed a lengthened mitotic cycle and a depressed respiration rate; these measurements display greater variability than our other mitotic cycle measurements, probably because the cultures were grown outside of an incubator and therefore were subject to greater variability in growth conditions.

In both sets of experiments exposed and control cultures were always in the same ambient laboratory environment or the same incubator; the possibility of the observed changes being caused by differences in incubators, including differences in temperature, airflow, etc. may be discounted. Furthermore, since the large incubator was three floors below and at the other end of the building from our usual location and since a location on still another floor was used for the non-incubator experiments, the possibility of our observations being caused by aberrations in the ambient electromagnetic fields or other peculiar conditions in our laboratory is also removed.

Blind Scoring of Mitosis

One criticism which has been made of our earlier reports was that the possibility of experimenter's bias existed in determining when mitosis occurred (NAS, 1977), since in most of our measurements the same technical personnel took the smears and read the slides. In one of the sets of experiments just discussed cultures exposed to 45 Hz, 3.0 G, 0.7 V/m EMF and con-

trol cultures were kept in a remote location outside of an incubator. Both mitotic cycle and respiration rate measurements were made by technical personnel who were unaware of the measured cultures' exposure regimen. Whenever a measurement was made, two randomly-coded flasks were brought to the laboratory, one containing an exposed culture and one, a control; the technician used these to set up and run the experiments in the usual manner. Only after a period of several months were the data collected and analyzed. As discussed above, the results shown in Table I indicate that the exposed cultures displayed the typical lengthening of the mitotic cycle and depression of the respiration rate.

Electrolysis Effects

The alternate hypothesis that our observed effects have been caused by electrolysis products, rather than by the fields themselves (NAS, 1977), has already been partially addressed in the reports of Goodman et al. (1976, 1979). The potentials placed across the stainless steel electrodes, which are in contact with the conducting growth medium (resistivity approximately 2 Ohm-meters) are small (40 mV at the highest field strength of 0.7 V/m and ranging down to 3 mV at the weakest fields). Long-lived electrolysis products are ruled out as a cause of effects by an experiment (Goodman et al. (1976) in which sterile growth medium was exposed to electric and magnetic fields before inoculation with Physarum; the cultures were then grown in control environments and no effects were noted. The possibility of short-lived electrolysis products was also partially addressed by Goodman et al. (1979) in cultures exposed to magnetic fields alone. In those experiments, our usual culture flasks incorporating stainless steel electrodes were used, but there was no voltage and hence no electric field or current between the electrodes. These measurements of

mitotic cycle length and respiration rate are summarized in Table I.

To further test the possible influence of electrolysis effects, two sets of experiments were conducted, each involving special growth vessels. To try to reduce the number of parameters in each test, one of these sets involved exposure to a magnetic field alone: the other, exposure only to an electric field. In the first set of experiments, a 75 Hz 2.0 G magnetic field was applied to cultures grown in all-glass vessels, similar to the usual ones except that the stainless steel electrodes were replaced by plain glass walls. Like the previous experiment that used only magnetic fields, no voltage or current effects could be present; in this instance any electrochemical or other effects due to the presence of the metal electrodes themselves was also eliminated. As indicated by Table I, the exposed cultures displayed depression in respiration rate and lengthened mitotic cycle, compared with control cultures grown in similar vessels. The mitotic cycle data are also presented in Figure 3, where we have plotted the frequency of observation of a particular change in cycle length in the exposed culture, relative to the average control culture cycle length for the day of observation.

In the other set of experiments cultures were exposed to electric fields that were produced by electrodes insulated from the growth medium. This set of experiments tested whether conduction currents, short-lived electrolysis products, or the presence of the stainless steel could be affecting the cultures. The special all-glass growth vessels for this set were similar to the usual design, except that the interior of two opposite walls was coated with a conducting layer of 100 Angstroms of vapor-deposited gold, over which an insulating layer of 20 Å of silicon oxide had been deposited

(Model CG-20AU-X coated glass plates, Practical Products Co., Cincinnati, Ohio). The DC resistance of these flasks, when filled with the conducting growth medium, was in excess of 10^6 Ohms, compared with about 30 Ohms for the usual vessels. The insulated electrodes were used to apply 0.1 V/m 75 Hz electric fields across the growth medium, as measured by a small dipole with uninsulated tips inserted into the medium and connected to a high impedance amplifier. The voltage applied to the insulated electrodes was 1.0 V (rms), which may be compared with approximately 6 mV which would have produced the same field strength using the stainless steel electrodes. Exposed cultures showed depressed respiration rates compared with control cultures grown in similar vessels; these data are listed in Table I. We therefore conclude that the stainless steel electrodes or their electrical contacts with conducting medium have not had an effect on the cultures which have been attributed to EMF.

CONCLUSION

The experiments in this paper supplement those reported earlier by this group to form a puzzling picture. Physarum and other living organisms (see for example, Tenforde (1978) and Phillips (1979)) are subtly affected by very weak electromagnetic fields. The mechanism of the interaction is not clear; however, there is some speculation that the mechanism involves some sort of membrane effect (Bawin et al. (1978) and Grodsky (1976)). Our earlier reported results confirm the subtlety of the effects since their initial detection occurs only after long exposure (Goodman, 1976). The present experiments tend to rule out a "genetic" mechanism. It is interesting that there is a persistent residual effect of exposure even though the observed effects have disappeared, as shown in the rapid reappearance of

effects in previously exposed cultures which were reintroduced into the fields. Further work is needed to localize the site(s) and biochemical system(s) that are involved in the interaction.

ACKNOWLEDGEMENTS

We wish to thank Dr. Joyce Mohberg for many useful discussions and for her assistance in preparing and photographing the cell nuclei. We also wish to acknowledge the skilled technical assistance of Mary Blanchard and Judith Swanson during the course of these experiments. Additional support was furnished by the UW-Parkside Committee on Research and Creative Activity.

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REFERENCES

Aronson, R. B., Van Slyke, D. D.: Manometric determination of CO_2 combined with scintillation counting of C-14. Anal. Biochem. 41, 173-183 (1971).

Bawin, S. M., Sheppard, A. R., Adey, W. R.: Possible mechanisms of weak electromagnetic field coupling in brain tissue. Bioelectrochem. and Bioenergetics, 5, 67-76 (1978).

Goodman, E. M., Beck, T.: Metabolism during differentiation in the slime mold Physarum polycephalum, Can. J. Microbiol., 20, 107-111 (1974).

Goodman, E. M., Greenebaum, B., Marron, M. T.: Effects of extremely low frequency electromagnetic fields on Physarum polycephalum. Radiat. Res. 66, 531-540 (1976).

Goodman, E. M., Greenebaum, B., Marron, M. T.: Bioeffects of extremely low frequency electromagnetic fields on Physarum polycephalum: variation with intensity, waveform and individual or combined electric and magnetic fields. Radiat. Res. (to be published, 1979).

Greenebaum, B., Goodman, E. M., Marron, M. T.: Long-term effects of weak 45-75 Hz electromagnetic fields on the slime mold Physarum polycephalum. In "Biological Effects of Electromagnetic Fields" (C. C. Johnson, and M. L. Shore, eds.), U. S. Department of Health, Education and Welfare Publication (FDA) 77-8010, Vol. 1, 449-459 (1977a).

- - -: Method for ensuring comparable temperatures in biological experiments using multiple incubators. Rev. Sci. Inst. 48, 937-938 (1977b).

Grodsky, I. T.: Neuronal membrane: A physical synthesis. Math. Biosci. 28, 191-219 (1976).

REFERENCES

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275 (1951).

Marron, M. T., Goodman, E. M., Greenebaum, B.: Mitotic delay in the slime mold Physarum polycephalum induced by low intensity 60 Hz and 75 Hz electromagnetic fields. Nature 254, 66 (1975).

McCullough, C. H. R., Cooke, D. J., Foxon, J. L., Sudbery, P. E., Grant, W. D.: Nuclear DNA content and senescence in Physarum polycephalum. Nature 245, 263-265 (1973).

Mohberg, J., Babcock, K. L., Haugli, F. B., Rusch, H. P.: Nuclear DNA content and chromosome number in the myxomycete Physarum polycephalum. Devel. Biol. 34, 228-245 (1973).

Mohberg, J., Rusch, H. P.: Isolation and DNA content of nuclei of Physarum polycephalum. Exp. Cell. Res. 66, 305-316 (1971).

NAS: Biologic effects of electric and magnetic fields associated with proposed Project Seafarer, U. S. National Academy of Sciences, Washington, D. C. (1977).

Pearson, E. S., Hartley, H. O.: Biometrika tables for statisticians. Biometrika Trust, London, Vol. 1, Sec. 5.7 and Table 10 (1976).

Tenforde, T. S., ed.: Proceedings of the Bioelectromagnetic Effects Workshop. Berkeley, Lawrence Berkeley Laboratory LBL-7452 (1978).

Phillips, R. S., ed.: Biological effects of extremely-low-frequency electromagnetic fields (Proceedings of the 1978 Hanford Life Sciences Symposium), Hanford, Washington, Battelle Northwest Laboratories (to be published, 1979).

REFERENCES

Winer, B. J.: Statistical Principles in Experimental Design. New York, McGraw-Hill, p. 28 (1962).

TABLE I. Means and standard deviations for mitotic cycle length and respiration rate experiments done to test alternative hypotheses.

Experimental and Field Conditions	Differences in Mitotic Cycle Length (2nd Metaphase, hr) Mean Difference	Std. Dev./N	Respiration Rate (QO_2, µl/min/mg protein) Control	Exposed	t/df[a]
Single Incubator in Basement 45 Hz, 2.0 G, 0.7 V/m	----	----	0.534	0.503	5.68/23*
Blind Scoring, No Incubator in Remote Room, 45 Hz, 3.0 G, 0.7 V/m	2.08*	1.40/22[b]	0.568	0.494	6.97/29*[b]
Magnetic Field Only (Stainless Steel Electrodes), 75 Hz, 2.0 G	0.44*	0.34/153	0.552	0.514	6.61/20*
Magnetic Field Only (All Glass) 75 Hz, 2.0 G	0.48*	0.39/130	----	----	----
Electric Field Only (Insulated Electrodes) 75 Hz, 0.1 V/m	----	----	0.569	0.518	6.17/47*

* $p < 0.01$

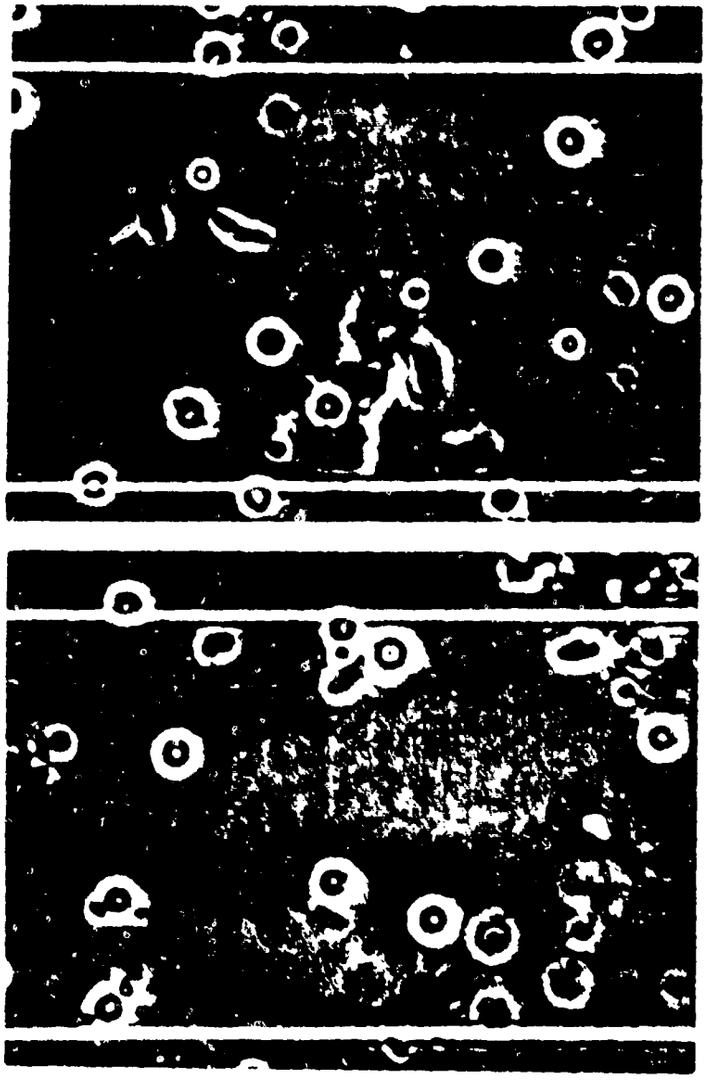
a The t-statistic is computed treating each day's experiment as paired data, control vs. exposed, minimizing effects of variation in the data due to small changes in external factors. The degrees of freedom (df) is the total number of data, less twice the number of days on which data were taken; three or four data points are taken on a given day.

b See discussion in text concerning increased variability due to lack of temperature control.

c Goodman et al. (1979).

FIGURE CAPTIONS

1. Photomicrographs of a) control Physarum nuclei and b) nuclei from cultures exposed to 75 Hz, 2.0 G and 0.7 V/m fields. Only images showing the bright "halo" were considered in focus enough to be measured. White lines are hemocytometer rulings with a spacing of 0.05 mm.
2. Difference in mitotic cycle length for exposed and control cultures of Physarum as a function of time after removal of the exposed cultures from the fields. Exposed cultures were placed in fields of 75 Hz, 2.0 G and 0.7 V/m, 1329 days before the start of this experiment. On the day indicated as "Day 0" in the Figure, exposed cultures were removed from the fields and placed in the control environment; they were replaced in the EMF environment 306 days later. Control cultures were always kept in an environment free of applied fields. Each point represents the difference between average time for ten exposed samples to reach the second metaphase and the average time for ten control cultures. The error bars are 95% confidence limits for that day, computed using a pooled estimate of the common variance (Winer (1962)).
3. Distribution of differences in mitotic cycle length, measured in hours, for cultures exposed to 75 Hz, 2.0 G, magnetic fields in all-glass vessels. The differences are taken relative to the average cycle length for each day's control samples.



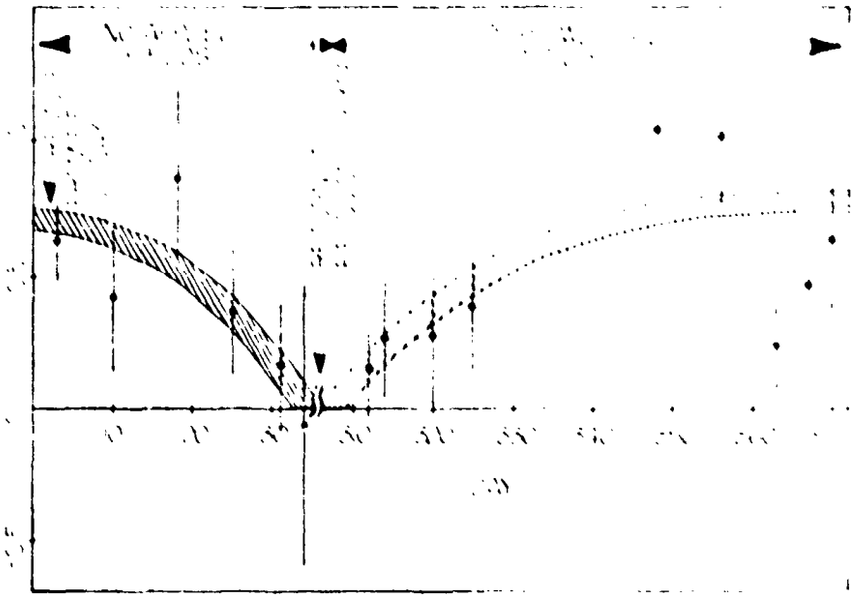


FIG. 2

6.2

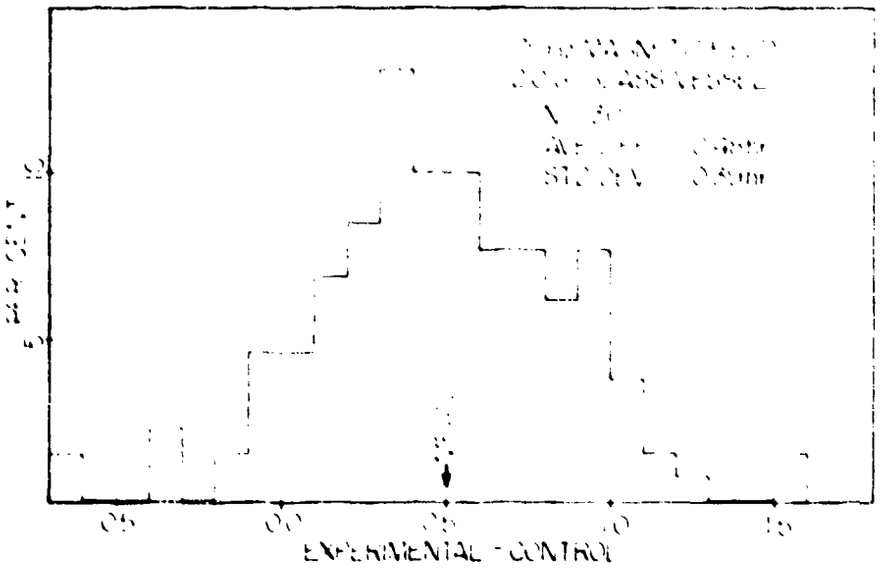


FIG. 3

Effects of Weak Electromagnetic Fields on Physarum polycephalum: Mitotic Delay in Heterokaryons

The results of mixing microplasmodia from both control and exposed cultures in equal volumes are shown in the table. After mixing, 1 single macroplasmodia is formed which undergoes synchronous mitosis at a time between those of the 2 parent cultures. This averaged behavior continues even when the mixture is predominantly non-exposed plasmodia, 3:1 by volume, or vice versa. In mixed (M) cultures made up 1 part control (C) to 3 parts exposed (E) culture, the average differences observed were 0.75 h (M-C) and 0.30 h (E-M). Comparable experiments with 3 parts control and 1 part exposed culture gave average differences of 0.27 h (M-C) and 0.79 h (E-M). These averages are computed from experiments involving 192 separate cultures and were conducted on 12 different days over a period of 1 year. The variation in these data is similar to that seen in the table for 1:1 mixtures. The length of the mitotic cycle in the mixture appears to depend on the amount of each type of culture in the mixture; however, the data are too sparse to determine a precise relationship.

Several investigators have previously shown that joining 2 existing macroplasmodia in different phases of the cell cycle can alter the timing of mitosis(1-3). The mixing experiments described here differ in that 1. contact between the 2 types of plasmodia occurs at the time the macroplasmodia are formed rather than after each individual macroplasmodium has established a synchrony of its own, and 2. the 2 partners do not have the same cycle lengths.

These experiments permit several conclusions to be drawn. First and foremost is that exposure of Physarum to weak electromagnetic fields produces biological effects. This conclusion is also supported by our

previous findings that exposure causes the mitotic cycle and protoplasmic shuttle streaming to slow. We may also conclude that the exposed cultures have been altered in some way that immediately manifests itself when control and experimental cultures are brought together. Similar averaging of the cell cycle has been reported by Haugli et al. (4) in mixtures of normal cultures and cell-cycle mutant cultures that have a lengthened cell cycle. A 3rd important conclusion is that the alteration brought about in exposed cultures is not drastic from the point of view of basic cellular processes. When exposed and unexposed microplasmodia are mixed together and allowed to fuse to form a single large microplasmodia, the fusion takes place and all nuclei behave normally to the extent that they undergo synchronous mitosis. They behave abnormally in that the mitotic cycle time is different from either parent culture. Since all of the processes we have observed to be influenced by EMF require energy, we suspect that weak low frequency EMF may interfere with either energy generating processes or the transport of essential metabolites in Physarum.

Summary

Continuous exposure of Physarum polycephalum to a 75 Hz, 2.0 G, and 0.7 V/m electromagnetic field results in a depressed respiration rate and a lengthening of the mitotic cell cycle. If unexposed Physarum are mixed with exposed Physarum the onset of synchronous mitosis in the mixted culture is delayed, occurring at a time between those of the 2 parent cultures.

References

1. H. P. Rusch, W. Sachsenmaier, K. Behrens and V. Gruter, J. Cell Biol. 31, 204 (1966).

2. E. Guttes, V. R. Devi and S. Guttes, Experientia 25, 615 (1969).

3. J. J. Wille and S. A. Kauffman, Biochem. biophys. Acta 407, 158 (1975).

4. F. B. Haugli, W. F. Dove and A. Jimenez, Molec. Gen. Genet. 118, 97 (1972).

TABLE I. TIME TO SECOND POST-FUSION MITOSIS FOR CONTROL, EXPOSED, AND MIXED CULTURES.

Mixed cultures are formed from equal volumes of control and exposed microplasmodia. In each row is given mean times in hours for cultures examined on a particular day. The number in parentheses next to the entry is the standard deviation of the mean. Entries in columns headed by a "Δ" are differences between the means appearing on either side of the entry; the average difference is given at the bottom of these columns. This table presents data taken for 290 separate cultures collected on 18 different days over a period of one year.

CONTROL	Δ	MIXED	Δ	EXPOSED
14.79 (.09)	.33	15.11 (.09)	.46	15.57 (.07)
15.67 (.10)	.24	15.92 (.14)	.66	16.58 (.06)
16.53 (.12)	.25	16.78 (.12)	.49	17.27 (.10)
16.11 (.11)	.24	16.35 (.10)	.67	17.02 (.06)
15.24 (.08)	.18	15.42 (.15)	.48	15.90 (.09)
15.37 (.02)	.02	15.39 (.05)	1.22	16.61 (.08)
15.39 (.08)	.17	15.56 (.04)	.33	15.89 (.03)
15.96 (.06)	.13	16.09 (.07)	.71	16.80 (.06)
15.08 (.05)	.50	15.58 (.05)	.16	15.74 (.03)
15.40 (.11)	.68	16.08 (.08)	.23	16.31 (.04)
15.76 (.04)	.15	15.91 (.07)	.88	16.79 (.03)
15.26 (.07)	.34	15.60 (.06)	.24	15.81 (.03)
16.07 (.03)	.40	16.47 (.03)	.16	16.63 (.08)
15.27 (.06)	.11	15.38 (.09)	1.25	16.63 (.10)
15.32 (.07)	.35	15.67 (.05)	.91	16.58 (.05)
15.45 (.14)	.21	15.66 (.11)	.58	16.24 (.04)
14.82 (.04)	.17	14.99 (.04)	.42	15.41 (.06)
15.38 (.08)	.17	15.55 (.04)	.88	16.43 (.07)
	.26		.60	

PRELIMINARY DATA

Based on our previous results on the EMF effects on Physarum and those of Bawin et al. (1978) with brain tissue, two additional experiments were undertaken to ascertain the EMF effects on calcium efflux and intracellular ATP.

CALCIUM EFFLUX

Materials and Methods

Microplasmodia were maintained as described previously. Experimental cultures were exposed to 45 Hz, 2.0 G, 0.7 V/m and displayed depressed O_2 consumption prior to their utilization in these experiments. At the beginning of an experiment microplasmodia from control and EMF environments were placed in growth medium to which exogenous calcium salts had been excluded. Microplasmodia from both control and experimental environments were allowed to grow in this medium for 72 hours whereupon the cultures were harvested and inoculated to 25 ml of the same calcium deficient growth medium containing a small amount of radioactive ^{45}Ca (1 μC/ml). Cultures were allowed to grow in the radioactive medium for an additional 48 hours. The ^{45}Ca labelled microplasmodia were harvested, washed three times in 10 volumes of growth medium and the plasmodial suspension was placed on filter paper supported by cotton sponges. After microplasmodia formed by coalescence of the microplasmodia, calcium deficient growth medium without isotope was added. At 15 minute intervals 0.2 ml aliquots of growth medium were withdrawn and the radioactivity determined.

Results and Discussion

Several experiments were performed using the protocols described above; data from a typical experiment is shown in Figure 1. Based on these experiments, it appears that the rate of ^{45}Ca efflux is greater

in control cultures than in EMF exposed cultures. The depressed calcium efflux data are somewhat similar to those reported by Bawin et al. (1978) when cerebral tissues from chickens and cats were subjected to similar EMF environments. These data represent the results of 7 experiments and although preliminary, they indicate that 45 Hz, 2.0 G, 0.7 V/m affects the movement of calcium.

REFERENCE

Bawin, S. A., A. Sheppard, W. R. Adey. Possible mechanisms of weak electromagnetic field coupling in brain tissue. Bioelectrochem. Bioenerg. 5, 67-76 (1978).

FIGURE LEGEND

Figure 1. A plot of ^{45}Ca efflux, 0 represents control cultures and □ represents cultures exposed to 45 Hz, 2.0 G, 0.7 V/m. These data have been acquired from three individual control and three individual EMF exposed cultures.

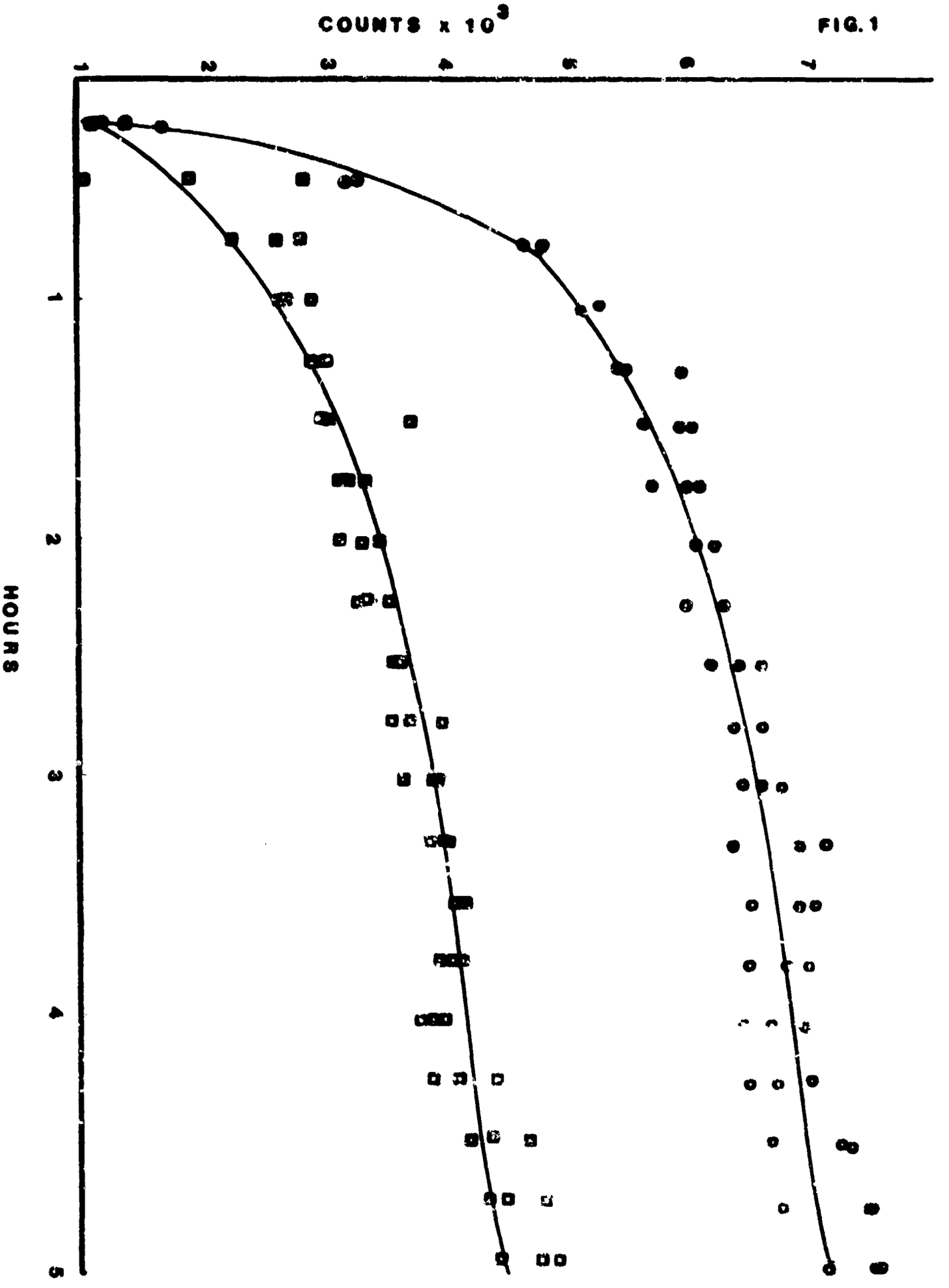


FIG. 1

INTRACELLULAR ATP

Materials and Methods

Microplasmodia from the control environment and ELF exposed (75 Hz, 2.0 G, 0.7 V/m) were harvested, centrifuged (250 xg), the growth medium was decanted, and the pellet was resuspended in 2 volumes of distilled water. Triplicate aliquots of each suspension (0.2 ml) were placed in 4.8 ml of Tris-borate buffer pH 9.2 (100°C) to extract the ATP. After a 5 minute extraction, the tubes were placed in an ice bath, followed by centrifugation 250 xg for 10^1 at 4° C. The supernatant was removed for ATP assay; nucleic acids were extracted from the pellet and the remaining residue was analyzed for protein. ATP was measured using the luciferin-luciferase assay in which the light emitted is proportional to the ATP concentration in the sample. The procedures described by Kimmich et al. (1975) were used to prepare the enzyme solution. Exogenous luciferin was added to insure maximum enzyme activity (Karl, Holm, Hanson, 1976). Measurement of emitted light was made using the photomultiplier assembly of a Turner fluorometer that had been interfaced to a Hewlett Packard electronic counter; samples were counted for 10 seconds.

Results and Discussion

Statistical analysis using a Fisher Sign test (M. Hollander and D. A. Wolfe, Nonparametric Statistical Methods, p. 39ff. (J. Wiley, New York, 1973).) show that at the P = 0.14 level, ATP concentrations in exposed cultures is greater than that in control cultures. Since we have customarily required a level of significance of at least P = 0.05 before we have concluded that an effect exists we regard these data as indicative but not conclusive.

COMPARISON OF ATP LEVELS IN CONTROL AND EMF EXPOSED CULTURES

Trial	Specific Activity* (ATP counts/ug protein) Control	Experimental
1	204	261
2	252	282
3	210	214
4	274	212
5	194	229
6	120	202
7	33	42
8	64	100
9	88	80
10	59	80
11	49	58
12	51	75
13	43	54
14	35	54
15	105	82
16	76	50
17	142	108
18	66.7	55
19	43	53

* Counts are proportional to the area under the curve of light intensity versus time.

REFERENCES

Kimmich, G. A., J. Randles, and J. S. Brand. Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. Anal. Biochem. 69, 187-206 (1975).

Karl, D. M., and O. Holm-Hansen. Effects of luciferin concentration on the quantitative assay of ATP using crude luciferase preparations. Anal. Biochem. 75, 100-112 (1976).

SUMMARY AND CONCLUSIONS

1. Exposure of Physarum polycephalum to extremely low frequency (ELF) electric (0.035 to 0.7 V/m) and magnetic fields (0.1 to 2.0 G) produces a decrease in respiration and nuclear division rates in the organism. These findings are consistent with results of earlier studies performed at different frequencies and field intensities.

2. Several sets of Physarum cultures have been exposed continuously for five years to 75 Hz fields of 0.7 V/m and 2.0 G. After an initial induction period the exposed cultures exhibited a mitotic cycle that was consistently longer than the control cycle by 0.6 hr. The induced increase in mitotic cycle length does not become progressively larger nor does the organism respond by compensating for exposure and slowly adjust its cycle length to agree again with the control cycle length. The observed decrease in respiration rate exhibits similar behavior.

3. Application of either a 75 Hz, 0.7 V/m electric field or a 75 Hz, 2.0 G magnetic field produces a decrease in growth and respiration rate; however, the effects are not as large as they are when both fields are applied simultaneously. Either field at these levels produces effects that are statistically indistinguishable from one another. We conclude that both electric and magnetic fields play a role in causing physiologic changes in the organism. It appears that these roles are additive when one examines the respiration data. The roles of the individual fields in slowing nuclear division are not additive: each field causes the rate to slow by about 0.4 hr but simultaneous application of the fields produces a cycle that is only 0.6 hr longer.

4. Exposure of Physarum to simultaneous 75 Hz electric and magnetic fields that are five times weaker (0.14 V/m and 0.4 G) produces effects that are statistically indistinguishable from those observed when either

a stronger electric field (0.7 V/m) or magnetic field (2.0 G) is applied by itself. These and other data suggest that the lower threshold for efficacy of one type of field may have been passed.

5. Frequency modulation of the applied electric and magnetic field produces bioeffects similar to those observed when unmodulated sinewave fields are applied. Although the data obtained are not completely self-consistent, we conclude that frequency modulation of ELF electromagnetic fields at these levels does not substantially alter the way ELF fields interact with biological systems.

6. No lower (or upper) threshold is observed for effects of ELF fields on Physarum. Electric fields were applied ranging from 0.035 V/m to 0.7 V/m; magnetic fields ranged from 0.1 G to 2.0 G. This finding contradicts one from an earlier report that ELF fields of 0.15 V/m and 0.4 G produce no effect in Physarum (11).

7. The dose-response relationship between field intensity and magnitude of the decrease in growth or respiration rate in Physarum is either very weak or nonexistent at the field intensities we have employed. A decrease of field intensities by a factor of four from 0.14 V/m and 0.4 G to 0.035 V/m and 0.1 G produces no significant difference in response. We attribute the large and significant difference in response observed when fields are reduced five times from 0.7 V/m and 2.0 G to 0.14 V/m and 0.4 G as being due to passing the lower threshold for one of the fields. Whichever field remains effective at the lower levels has a very flat dose-response curve.

8. If unexposed Physarum microplasmodia are mixed with EMF exposed Physarum microplasmodia, the onset of synchronous mitosis in the fused macroplasmodia is delayed. The magnitude of the mitotic delay occurs

at an intermediate time between those of the two parent control cultures.

9. The National Academy of Science review of our experiments raised two questions concerning 1) the absence of blind scoring mitosis and 2) the possibility that short-lived electrolysis products may have induced the physiological effects we have observed. A series of blind experiments on both respiration and the mitotic cell cycle have shown a depression in O_2 consumption and a lengthened cell cycle in EMF exposed cultures (45Hz, 3.0G, 0.7V/m). Electrolysis as a cause of these effects has been ruled out by experiments in which altered physiological responses were observed in cultures exposed to EMF in flasks with silicon oxide coated electrodes. Similar effects were also observed using all glass vessels subjected only to magnetic fields.

10. Several non-EMF factors such as incubator differences, divergence in chromosome number, and ambient electromagnetic fields have been ruled out as possible sources of observed effects.

11. Preliminary data indicate that ATP levels in EMF-exposed cultures are elevated relative to non-exposed cultures. Studies on calcium efflux suggest that the plasma membrane of control cultures is more permeable to calcium efflux than that of EMF exposed cultures.

RECOMMENDATIONS

We have pointed out in several places that many, fundamental questions have been raised by the work carried out under this contract. All of these deserve further study to understand the basic processes involved in the bioeffects of EMR. There are a few broad and pressing question, however, that deserve further investigation.

1. Refinement of ATP and Calcium Transport Studies. During the last stages of the contract we developed procedures to examine ATP levels and Calcium efflux in Physarum exposed to ELF. Preliminary findings indicate that ATP levels in exposed cultures may be greater than those in control cultures and that calcium ions leak out of exposed Physarum at a slower rate compared to control cultures. Definitive statements on these points cannot be made without improved experimental precision. Because of the fundamental importance of both ATP levels and calcium transport to all cell processes, these experiments should be improved, repeated, and elaborated.

2. Examination of Comparable Field and Laboratory Exposures. Our studies were designed to examine the effect of a single factor, namely, exposure to ELF fields. This, of course, makes good sense from a scientific point of view but it presents certain difficulties (often hidden) to those seeking to translate our findings into meaningful terms for assessing potential hazards of an ELF transmitter. In simple terms the difficulty may be stated in the form of a question: When an organism is moved from pristine laboratory conditions to a situation where it is buffeted by variations in factors such as temperature and humidity, are the effects of ELF fields enhanced or diminished? Because the basic mechanism of interaction between ELF fields and biological systems is not known, it is virtually impossible to answer this question without the benefit of empirical evidence. We believe that observations should be performed on

organisms exposed to ELF fields in the environment and that these observations should be compared with those made on organisms exposed to comparable fields in the laboratory. Furthermore, because of the similarity of fields under power transmission lines to those anticipated near an ELF antenna (cf. National Academy of Sciences Report, p. 118) this type of an experiment could easily be conducted either at the Clam Lake site or near power transmission lines. Since transmission lines are energized continually under fairly constant loads, the latter setting would seem to offer several advantages.

3. Rule Out the Large Size of Physarum Microplasmodia as a Contributing Factor to Observed Effects The National Academy of Sciences Report suggested that because Physarum microplasmodia are as much as ten times larger than some mammalian cells they may exhibit an extraordinary sensitivity to ELF fields. This hypothesis should be tested by exposing P. amoebae to one or two selected ELF fields. P. amoebae are morphologically and physiologically similar to mammalian cells in all respects except that they are much simpler to raise in culture for extended periods of time.

To one acquainted with the subtleties of experimental design this might seem at first blush to be a foolish proposal to make. Would not moving the cultures outdoors make observations due to EMF exposure more difficult to observe due to variations caused by uncontrolled factors, and thus make observation of any effects due to ELF more difficult? The answer is no. We are suggesting a test of the hypothesis that variations in external factors other than ELF fields, have a synergistic effect on the interaction of ELF fields with biological organisms. It may be that the major stress of occasional extreme temperature/humidity/geomagnetic field/etc. variations may overwhelm an organism to the point that minor

to raise in culture for extended periods of time.